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BIOACTIVE METABOLITES OF *ALTERNARIA BRASSICAE* AND *MONOCILLIUM NORDINII*

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by

LUIS MANUEL PEÑA RODRIGUEZ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

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Supervisor

External Examiner

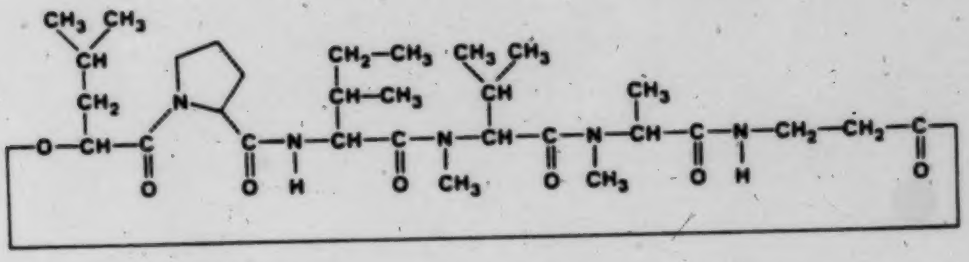
DATE October 11 1985

A mis padres, mis hermanos y la memoria de mis abuelitos

ABSTRACT

Alternaria brassicae is the fungus responsible for the "black spot" disease in canola (rapeseed) plants. The disease manifests itself most visibly on the leaves where it appears as a thin black spot known as the "necrotic center" surrounded by a yellow halo or "chlorotic zone". Part I of this thesis describes the isolation and identification of some of the metabolites present in the broth of A. brassicae when grown in a V-8 juice medium.

One of the compounds responsible for inducing chlorosis, as determined by biological testing (leaf-spot bioassay), has been identified as destruxin B (**1**). The structurally related cyclodepsipeptides desmethyldestruxin B (**2**) and homodestruxin B (**3**), as well as the drimane ses-



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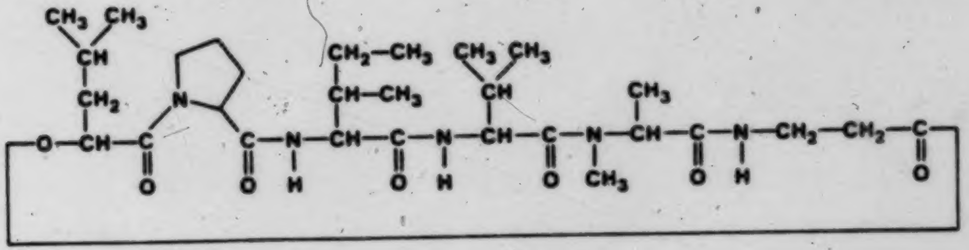

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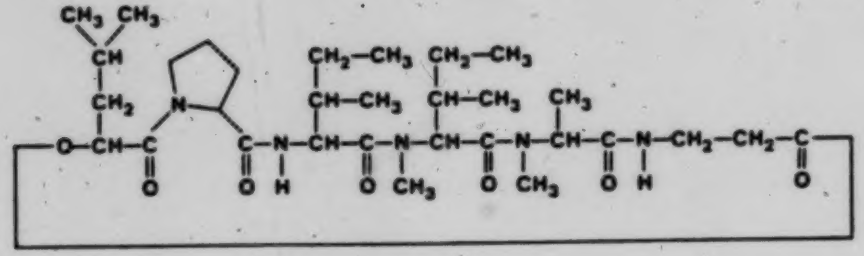

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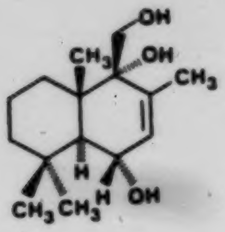
quiterpenes albrassitriol (4), isoalbrassitriol (5), deoxyuvidin B (6), and the biogenetically interesting brassicadiol (7) are also produced by the fungus. Compounds 3, 4, 5, 6, and 7 are new. Their structures have been established by physical and chemical methods.



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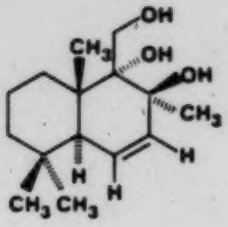

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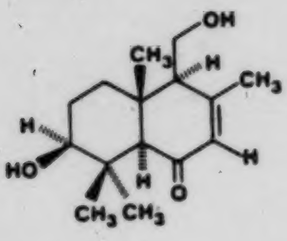

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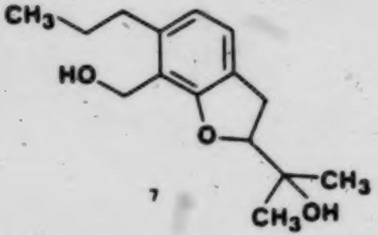

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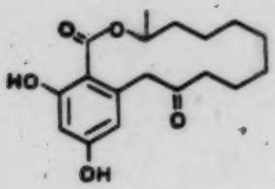
Part II of the thesis describes research carried out on the fungus Monocillium nordinii, a destructive mycoparasite of pine stem rust fungi. Examination of the metabolites produced in liquid culture by M. nordinii resulted in the isolation and characterization of the new compounds nordinone (**8**) and nordinonediol (**9**), as well as the known compounds sterigmatocystin (**10**), monorden (**11**), and monocillins I (**12**), II (**13**), III (**14**) and IV (**15**).



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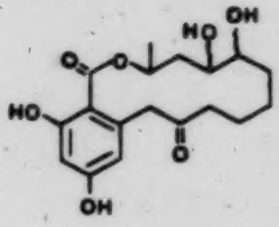
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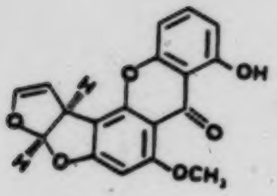
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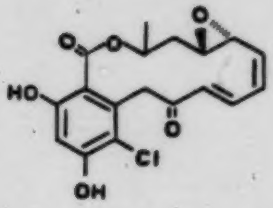
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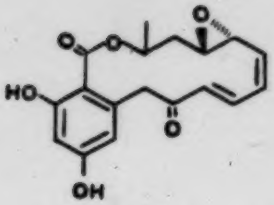

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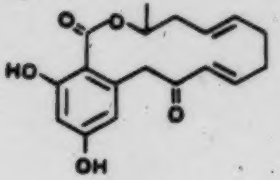

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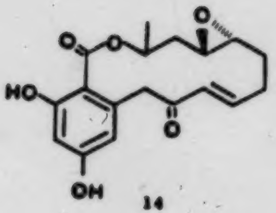

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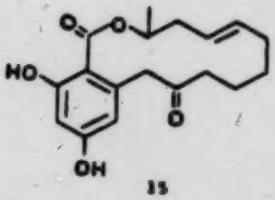

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Chemical modifications of monocillin I (**12**) and a study of the growth of *M. nordinii* under different culture conditions are also discussed.

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TABLE OF CONTENTS

PAGE

STUDY I... 1

INTRODUCTION...................................... 2

RESULTS AND DISCUSSION............................ 14

EXPERIMENTAL...................................... 99

Work up and Extraction of Metabolites............ 103

Preliminary Fractionation of the Crude Extracts... 105

Isolation and Identification of Metabolites....... 108

Appendix of Experimental......................... 131

STUDY II.. 133

INTRODUCTION...................................... 134

RESULTS AND DISCUSSION............................ 138

Part I. Isolation and Identification of Metabolites.......................... 139

Part II. Chemical Modification of Monocillin I (2).......................... 163

Part III. Growth of Monocillin nordinii Under Different Conditions and HPLC Analysis of the Crude Broth Extracts............ 170

EXPERIMENTAL...................................... 182

Isolation and Identification of Metabolites....... 189

Appendix of Experimental......................... 203

REFERENCES - STUDY I.............................. 204

REFERENCES - STUDY II............................. 212

LIST OF TABLES

TABLE PAGE

I. Alternaria alternata HST's known to date...... 7

II. Summary of Alternaria metabolites............ 10

III. Fragment ions in the HRMS of **A** and its methylation product.......................... 18

IV. Major fatty acids contained in **A**.............. 21

V. Chemical shifts of the protons of compound **E**...................................... 42

VI. Spin systems of compound **E**.................. 43

VII. C-13 values of carbons in compound **E**.......... 47

VIII. Fragment ions in the HRMS of **E'** and **E"**........ 52

IX. Typical MS fragmentations in a cyclodepsipeptide having an N-methylvalyl-N-methylalanyl moiety...................... 54

X. Destruxins reported to date.................. 56

XI. Fragment ions in the HRMS of destruxin B (**13**) and homodestruxn B (**16**)................ 58

XII. Fragment ions in the HRMS of the methylester derivatives of destruxin B (**12**) and homodestruxin B (**17**)..................... 61

XIII. Fragment ions in the HRMS of the methylester derivatives of destruxin B (**12**) and desmethyldestruxin B (**19**)................ 66

XIV. C-13 values of carbons in albrassitriol (**26**)...................................... 74

LIST OF FIGURES.

FIGURE PAGE

STUDY I:

1. Host-parasite interaction in plant diseases.. 5

2. Chromatogram of **A**-methylester................. 19

3. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of cerevisterol (**2**).............................. 23

4. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3/C\underline{D}_3O\underline{D}$) of β-sitosteryl-β-D-glucoside (**6**)............... 29

5. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of O,O,O,O-tetraacetyl-β-sitosteryl-β-D-glucoside (**C'**).. 31

6. IR spectrum ($CHCl_3$ cast) of destruxin B (**13**).. 38

7. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of destruxin B (**13**).............................. 40

8. APT ^{13}C NMR spectrum ($C\underline{D}Cl_3$) of destruxin B (**13**).. 48

9. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of homodestruxin B (**16**)........................... 60

10. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of desmethyldestruxin B (**18**).................... 64

11. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of albrassitriol (**26**).......................... 68

FIGURE PAGE

12. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of O,O-diacetylalbrassitriol (**D'**)........................ 70

13. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of isoalbrassitriol (**28**)........................ 77

14. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of deoxyuvidin B (**30**)........................ 82

15. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of brassicadiol (**38**)........................ 90

16. APT ^{13}C NMR spectrum ($CDCl_3$) of brassicadiol (**38**)........................ 91

STUDY II:

1. 100 MHz ^{1}H NMR spectrum ($CDCl_3$) of sterigmatocystin (**8**)........................ 141

2. 400 MHz ^{1}H NMR spectrum (CD_2Cl_2/CD_3OD) of ergosterol endoperoxide (**9**)........................ 144

3. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of monorden (**1**)........................ 149

4. 200 MHz ^{1}H NMR spectrum ($CDCl_3$) of monocillin I (**2**)........................ 153

5. 400 MHz ^{1}H NMR spectrum (CD_2Cl_2) of monocillin III (**4**)........................ 154

6. 400 MHz ^{1}H NMR spectrum (CD_2Cl_2) of monocillin IV (**5**)........................ 157

FIGURE PAGE

7. 400 MHz ^1H NMR spectrum ($C\underline{D}Cl_3$) of nordinone (**14**) 159

8. 400 MHz ^1H NMR spectrum (DMSO-d_6) of nordinonediol (**15**) 161

9. HPLC Chromatogram of monorden (**1**) 173

10. HPLC chromatogram of monocillin I (**2**) 174

11. HPLC chromatogram of the PDB containing 0.2% yeast crude 175

12. HPLC chromatogram of the crude from PDB no Cl^- 176

13. HPLC chromatogram of the crude from PDB, no Cl^-, but added Br^- 177

14. HPLC chromatogram of the crude from PDB, no Cl^-, but added F^- 178

15. HPLC chromatogram of the crude from "Homemade" 179

16. HPLC chromatogram of the crude from "Homemade" + Cl^- 180

LIST OF FLOW DIAGRAMS

DIAGRAM PAGE

1. *A. brassicae* (modified Czapek-Dox, 60 days.... 104

2. *A. brassicae* (V-8 juice medium, 20 days)...... 106

LIST OF SCHEMES

SCHEME PAGE

STUDY I

I. McLafferty rearrangement of straight-chain monocarboxylic acids and esters............... 17

II. MS fragmentation of C-17 substituted steroids.................................. 25

III. Loss of ring A in the MS of 3-hydroxylated steroids.................................. 26

IV. Loss of ring A in the MS of cerevisterol (**2**)...................................... 27

V. Ring C cleavage in the MS of cerevisterol (**2**)...................................... 27

VI. Fragmentation pattern in the MS of linear polypeptides............................. 50

VII. MS Fragmentation of compound **G**............... 80

VIII. Cyclization of epoxyfarnesylpyrophosphate (**32**)..................................... 85

IX. Epoxidation of deoxyuvidin B (**30**)............ 88

X. Possible biogenesis of compound **I**............ 96

XI. Possible biogenesis of compound **I**............ 96

XII. MS fragmentation of compound **I**................ 98

STUDY II.

I. MS fragmentation of ergosterol endoperoxide (**9**)...................................... 143

STUDY I

"We see our cattle fall and our plants wither
without being able to render them assistance,
lacking as we do understanding of their
condition". Fabricius (1774)

INTRODUCTION

In addition to insect and weed pests, another major cause of the destruction of plants is disease. Infectious diseases of plants are caused most commonly by fungi, bacteria and other microorganisms. It is known that these phytopathogens often produce toxic components, referred to as "toxins" or "phytotoxins",* that cause the disease symptoms.

Early reports on phytotoxins focused mainly on their biological activity rather than their chemistry or mode of action, since it was believed that they were of limited importance in pathogenesis and led only to mechanical injury on host plants leading to the disease symptoms. During the past two decades the toxin concept has changed significantly due to the intense activity in the field, and is now known that toxins do have a definite role, either in the initial attack and colonization or in that they exert their effects later, once the colonization has begun.

---

*For reviews of phytotoxins, their isolation, assay, structure elucidation and role in pathogenesis see references 1-8.

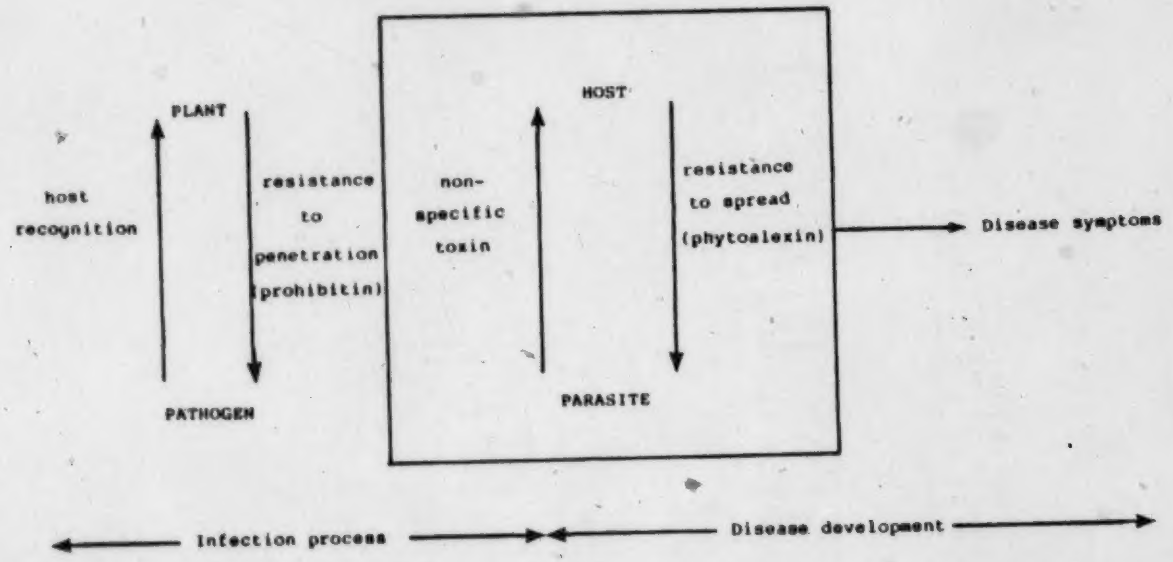
The present era of phytotoxin studies was initiated after the destructive epidemics of Victoria blight of oats (*Helminthosporium victoriae* Meehan and Murphy) and southern corn leaf blight (*Helminthosporium maydis* Nisikado and Miyake) in the United States, as well as the epidemics of apple blotch (*Alternaria alternata* (Fries) Keissler apple pathotype or *Alternaria mali* Roberts) and black spot of pears (*Alternaria alternata* pear pathotype or *Alternaria kikuchiana* Tanaka) in Japan showed the dramatic importance of toxins in plant diseases.[1,2] In these cases the importance of biologically active compounds with remarkable specificity was demonstrated.

This resulted in the classification of phytotoxins into two groups:[1,5] host-specific and non-specific toxins. Host-specific toxins (HST's) are defined as metabolic products of pathogenic microorganisms which are toxic only to the host plants of these pathogens. They represent widely different chemical categories, e.g., peptides, glycosides, linear polyketols and esters, and are said to be primary determinants of phatogenicity.

Non-specific toxins include plant growth regulators, mycotoxins, and other biologically active compounds which are not essential for pathogenicity but contribute to virulence. For this reason they are known as secondary determinants of pathogenicity. A representative host-parasite interaction, including the roles played by the toxins, is shown in Figure 1.

Due to their important role in pathogenesis and the information that they can provide regarding the molecular basis of plant disease, HST's have been the subject of extensive multidisciplinary studies in recent years.[1,2,4,5] The nature of the work has dealt mainly with the isolation and characterization of the compounds as a prerequisite to locating the site of action of the toxin and establishing how the toxin-target interaction functions. The importance of this structural information and of the availability of pure compounds cannot be overemphasized since these provide the "keys" which can allow the exploitation of many other areas of toxin research, e.g. biosynthesis, mechanism of action, metabolic regulation, structure-activity relationships, and metabolism. Synthesis of the toxin provides unequivocal proof of the structure and allows the preparation of specifically labeled material. It has facilitated the preparation of analogs which may be useful in the study of structure-activity relationships.

The present knowledge of HST's has come almost


PLANT
PATHOGEN
host recognition
resistance to penetration (prohibitin)
non-specific toxin
HOST
PARASITE
resistance to spread (phytoalexin)
Disease symptoms
Infection process
Disease development

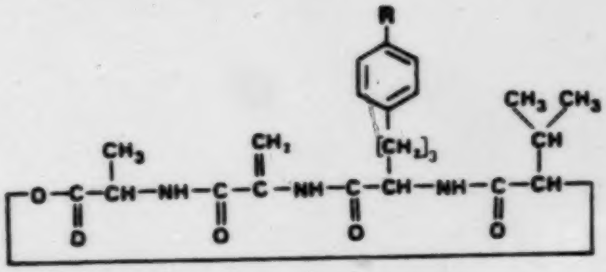
Ref. 11

Figure 1. Host-parasite interaction in plant diseases.

entirely from the so-called "saprophytic pathogens". Included are familiar fungi such as *Alternaria*[9-14] and *Helminthosporium*[15,16] and others that are less well known. Curiously, most of these appeared to be causes of so-called "man-made disease", diseases which occur only on newly bred or newly introduced cultivars of crop plants. Work on HST's produced by *Alternaria* began only about 15 years ago and so the present status of knowledge is still quite primitive.

Alternaria alternata displays a number of pathotypes[9] that affect certain plant species, each producing HST's primarily responsible for symptom production in the infected host plant. The six presently known host-parasite combinations for *Alternaria* pathogens where the toxins produced in culture display the same host specificity as that of the pathogen itself are listed in Table I.

The structure of the HST produced by the apple pathotype, AM-toxin I (**1**) was the first to be completely



1

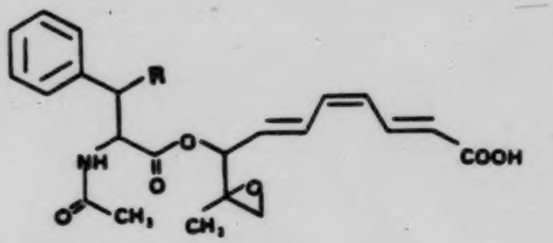
AM-toxin I R = $-OCH_3$
AM-toxin II R = H
AM-toxin III R = OH

Table I.* *Alternaria alternata* HST's known to date (1982).

Pathotype of *A. alternata* (previous name)	Disease	Host	Toxin
Apple pathotype (*A. mali*)	Alternaria blotch of apple	Apple, pear	AM-toxin
Citrus pathotype (*A. citri*)	Brown spot of citrus	Citrus	AC-toxin
Japanese pear pathotype (*A. kikuchiana*)	Black spot of Japanese pear	Japanese pear	AK-toxin
Strawberry pathotype	Alternaria black spot of strawberry	Strawberry, Japanese pear	AF-toxin
Tobacco pathotype (*A. longipes*)	Brown spot of tobacco	Tobacco	AT-toxin
Tomato pathotype (*A. alternata* f.sp. *lycopersici*)	Stem canker of tomato	Tomato	Al-toxin

*Ref. 9

elucidated[10,11,17-21] and proven by chemical synthesis.[22,23] More recently the structures of two other HST's, those of the AK- (**2**)[10,24] and AL-toxins (**3**)[25,26], have been assigned.



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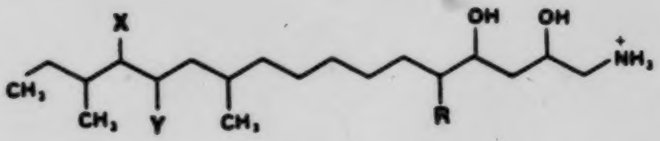
AK-toxin I R = CH_3
AK-toxin II R = H



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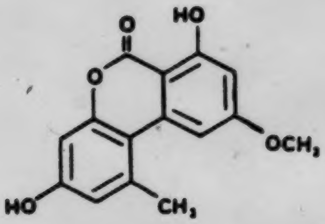
AL-toxin		R	X	Y
T_A	a	OH	OH	$^-OOC-CH_2-(COO^-)-CH_2-COO^-$
	b	OH	$^-OOC-CH_2-(COO^-)-CH_2-COO^-$	OH
T_B	a	H	OH	$^-OOC-CH_2-(COO^-)-CH_2-COO^-$
	b	H	$^-OOC-CH_2-(COO^-)CH_2COO^-$	OH

A significant number of non-specific toxins showing a wide range of disease symptoms and diverse chemical properties have been isolated from culture filtrates of several phytopathogenic Alternaria species. They are all non-enzymatic, low molecular weight compounds, ranging from small peptides to simple phenols. A number of them are listed in Table II. Among the most important are alternariol methylether (**4**),[9] produced by several pathogens, which is a causative agent of necrosis on pear leaves and chlorosis on tobacco leaves. Tenuazonic acid (**5**)[9] is a phytotoxic tetramic acid not unique to Alternaria fungi. It is known to produce necrotic lesions with or without chlorosis in plants. The cyclic tetrapeptide tentoxin (**6**)[27-30] is produced by Alternaria tenuis Nees and is reported to cause strong chlorosis in cotyledons of some plant species.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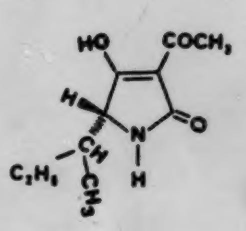
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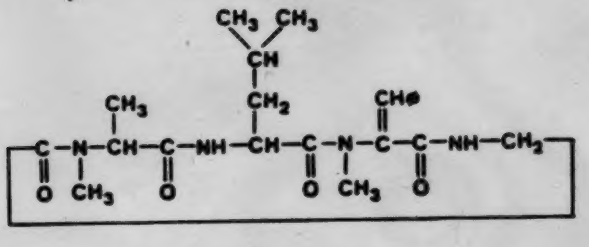

6

One species of Alternaria that has not received much attention is Alternaria brassicae (Berkeley) Saccardo, the fungus responsible for the "black spot" disease of canola

Table II.* Summary of Alternaria metabolites.

Metabolite	Species[a]	Molecular Formula
Alternaric acid	1	$C_{21}H_{30}O_8$
Phytoalternarin A	2	
Phytoalternarin B	2	
Phytoalternarin C	2	
Alternariol	3,5,6	$C_{14}H_{10}O_5$
Alternariol methylether	3,5,6	$C_{15}H_{12}O_5$
Alternenuol	3	$C_{14}H_{10}O_6$
Alternurin	3	$C_{15}H_{14}O_6$
Dehydroaltenurin	3	$C_{15}H_{12}O_6$
Altenuic acid I	3	$C_{15}H_{14}O_8$
Altenuic acid II	3	$C_{15}H_{13}O_8$
Altenuic acid III	3	$C_{15}H_{14}O_8$
Tenuazonic acid	3	$C_{10}H_{15}NO_3$
Tentoxin	3	$C_{22}H_{30}N_4O_4$
Altenin	1	$C_9H_{14}O_6$
Zinniol	4	$C_{15}H_{21}O_4$
Radicinin	7	$C_{12}H_{12}O_5$
Radicinol	7	$C_{12}H_{14}O_5$
Deoxyradicinin	8	$C_{12}H_{12}O_4$
Solanapyrone A	1	$C_{18}H_{22}O_4$
Solanapyrone B	1	$C_{18}H_{24}O_4$
Solanapyrone C	1	$C_{19}H_{25}NO_4$

[a]Key: (1) A. solani; (2) A. kikuchiana; (3) A. tenuis; (4) A. zinniae, (5) A. cucumerina; (6) A. dauci; (7) A. chrysantheni; (8) A. helianthi.

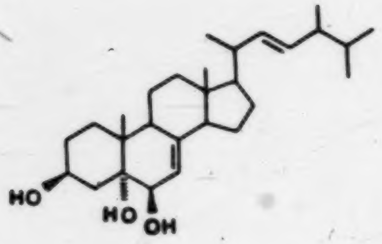
*Ref. 9

(rapeseed) plants,[12] an important crop for oilseed production in Western Canada.[31] It has been reported that when canola plants are infected with A. brassicae there is a substantial reduction in seed yield and quality of the oil.[12]

The pathogen attacks all parts of the plant, particularly the leaves, where it causes lesions consisting of a black necrotic center surrounded by a yellow halo or chlorotic zone. It is known that the latter effect is induced by a toxin or mixture of toxins.[12]

Professor J.P. Terwari of the Department of Plant Science of this university brought the "black spot" disease to our attention and since very little chemical work has been reported we set out to isolate and identify the toxic components produced by Alternaria brassicae. The availability of the disease-causing compounds, as was mentioned previously, will facilitate the biological study of the disease.

The fungus was grown in liquid "still" culture on a V-8 Juice-containing media for twenty days and the mycelial mat was removed by filtration. The culture filtrate was extracted with ethyl acetate and from this organic crude extract several metabolites, including cerevisterol (**7**) and β-sitosteryl-β-D-glucoside (**8**), were



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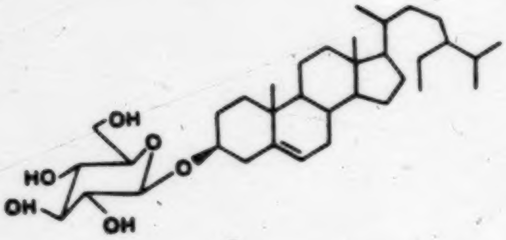
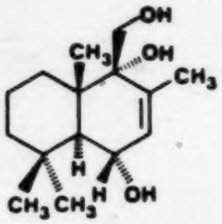
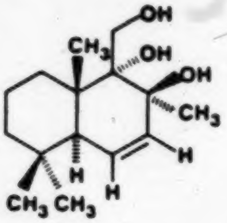

OH
O
HO
OH
OH
O

8

isolated and identified. However, these initial separations usually afforded complex mixtures difficult to separate. This problem was solved by partitioning the organic extract between different solvents. Purification of the medium polarity fraction afforded the previously unreported drimane sesquiterpenes albrassitriol (**9**), isoalbrassitriol (**10**) and deoxyuvidin B (**11**), together with the novel natural product brassicadiol (**12**).


OH
CH_3
OH
CH_3
H
CH_3 CH_3
H
OH

9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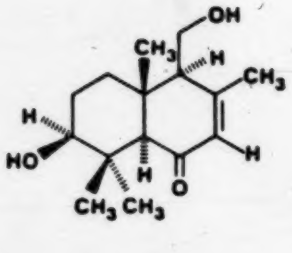
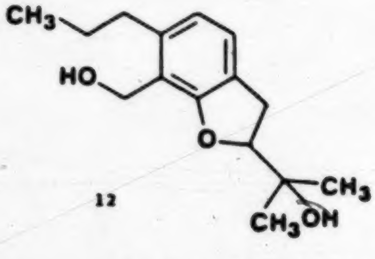
CH_3
OH
OH
CH_3
H
H
CH_3 CH_3
H

10

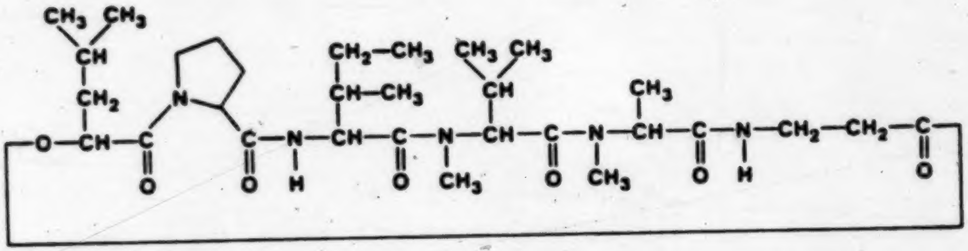

OH
CH_3
H
CH_3
H
HO
H
H
O
CH_3 CH_3

11


CH_3
HO
O
CH_3
CH_3
OH

12

From the same fraction three cyclodepsipeptides were isolated and identified. Of the three, the one that possesses the strongest chlorosis-inducing activity and which is produced in larger amounts has been identified as destruxin B (**13**), an insecticidal metabolite also produced by Metarrizium anisopliae (Metschnikoff) Sorokin.[32,33] The remaining two cyclodepsipeptides have been identified as homodestruxin B (**14**) and desmethyldestruxin B (**15**),[33,34] the former not having been reported previously. These results are discussed in detail in the following section.



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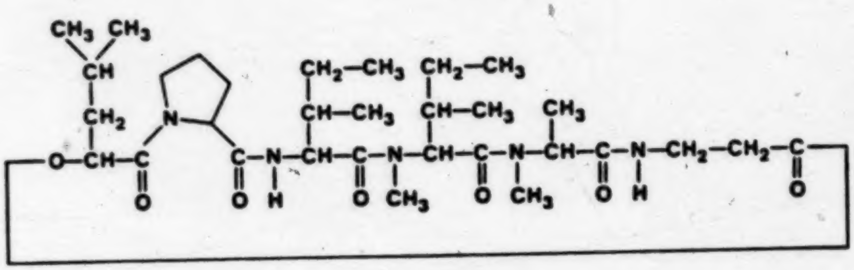

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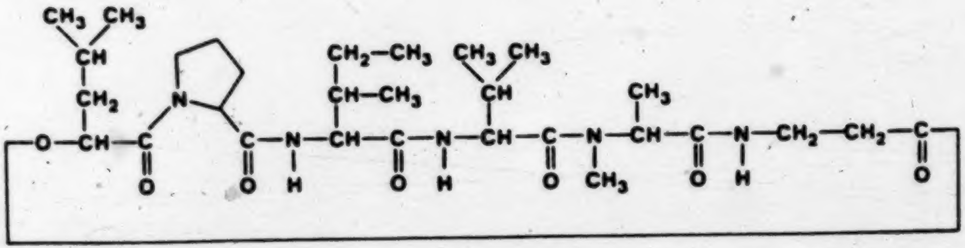

15

RESULTS AND DISCUSSION

Our work began with the investigation of the growth of Alternaria brassicae (UAMH 4936)[*] in three different culture media: modified Czapek-dox media, V-8 juice media and V-8 juice containing ca. 0.05% rose bengal.[**] Rose bengal was added to the media since it has been reported to stimulate sporulation of A. brassicae.[35] The three media were compared on the basis of maximum production of metabolites, ease of work up, and the ability of the culture filtrates and corresponding organic extracts[***] to induce chlorosis on canola leaves.

The main problem encountered when the fungus was grown in V-8 juice medium containing rose bengal was the presence of the red pigment in the culture filtrate, the mycelial mat, the organic extracts and on the equipment.

Extraction of the fungus grown on modified Czapek-dox medium gave syrupy culture filtrates which produced heavy emulsions on extraction and a lower yield of crude metabolites. V-8 juice medium was chosen for production of the Alternaria brassicae metabolites since it gave reasonable yields and did not present problems in the extraction.

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[*] Assession number given to Alternaria brassicae upon deposition at the University of Alberta mold herbarium.

[**] For composition see Appendix of Experimental.

[***] For detailed extraction procedure see Experimental.

Biological activity[*] was observed in the culture filtrates obtained from the three fungal growths when tested on canola leaves using the leaf-spot bioassay.[**] The strongest activity was observed for the culture filtrates of A. brassicae grown on V-8 juice medium.

A major problem encountered in this research was the need for a reliable bioassay in order to detect the presence of biological activity in the crude organic extracts obtained from the different fungal cultures.

One problem encountered in designing a bioassay arose from the poor solubility of the crude extracts in water. When the crude extracts were dissolved in organic solvents and the leaf-spot bioassay was attempted, the solvent evaporated before any effect exerted by the metabolites could be seen. The fact that the toxic component or components were present in the organic extract could easily be confirmed by the fact that the biological activity present in the aqueous culture filtrates was lost after extraction of the filtrate with ethyl acetate. While the bioassay problems were being studied, preliminary chemical investigations of the mycelial extract of A. brassicae grown on modified Czapek-dox medium were undertaken. Separation of this crude extract was accomplished by silica gel column chromatography using a gradient elution.* A total of 21 fractions, most of them mixtures, was obtained. From these fractions three components labeled **A**, B and C, listed in order of increasing polarity, were purified by further chromatographic separations.

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[*] Biological activity and ability to induce chlorosis will be used interchangeably.

[**] For description see Experimental.

Compound **A** was the most abundant component in the mycelial extract as revealed by thin layer chromatography (TLC).** The infrared (IR) spectrum of compound **A** shows broad and intense hydroxyl absorption (3400-2400 cm^{-1}) as well as carbonyl absorption (1708 cm^{-1}), both characteristic of a carboxylic acid.[36,37]

The presence of a carboxylic acid functionality is supported by the high resolution mass spectrum (HRMS) of **A** which shows a fragment of m/z 60 resulting from McLafferty rearrangement (Scheme I), a fragmentation characteristic

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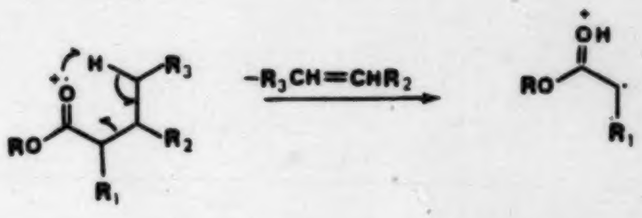
*For a detailed description see Experimental.

**Unless specified, all references to TLC behavior shall imply silica gel as the adsorbent and 30% H_2SO_4 followed by charring as the visualization method.

Scheme I

McLafferty Rearrangement of Straight-chain Monocarboxylic Acids and Esters



R = R_1 = H   m/z 60

R = CH_3, R_1 = H   m/z 74

of straight-chain monocarboxylic acids.[36] However, the series of high mass peaks observed in the HRMS and listed in Table III suggested that **A** was a mixture of fatty acids. The methyl ester derivatives of **A** were prepared by treatment of **A** with ethereal diazomethane, and the methyl ester mixture, **A'**, was analyzed by gas chromatography*-mass spectrometry (GC-MS). Five major methyl esters were observed (Figure 2) in the gas chromatogram. Each ester

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*Column used: Apiezon L (10%), for conditions see Experimental.

Table III. Fragment ions in the HRMS of **A** and its methyl ester.

A		**A'**	
m/z (int. %)	Formula	m/z (int. %)	Formula
382 (4)	$C_{25}H_{50}O_2$	396 (2)	$C_{26}H_{52}O_2$
368 (78)	$C_{24}H_{48}O_2$	382 (41)	$C_{25}H_{50}O_2$
354 (34)	$C_{23}H_{46}O_2$	368 (19)	$C_{24}H_{48}O_2$
340 (100)	$C_{22}H_{44}O_2$	354 (58)	$C_{23}H_{46}O_2$
326 (7)	$C_{21}H_{42}O_2$	340 (4)	$C_{22}H_{44}O_2$
312 (20)	$C_{20}H_{40}O_2$	326 (8)	$C_{21}H_{42}O_2$
284 (3)	*$C_{18}H_{36}O_2$	298 (1)	$C_{19}H_{38}O_2$
282 (5)	$C_{18}H_{34}O_2$	297 (2)	$C_{19}H_{37}O_2$
256 (1)	$C_{16}H_{32}O_2$	270 (1)	$C_{17}H_{34}O_2$
73 (50)	$C_3H_5O_2$	87 (72)	$C_4H_7O_2$
60 (37)	$C_2H_4O_2$	74 (100)	$C_3H_6O_2$

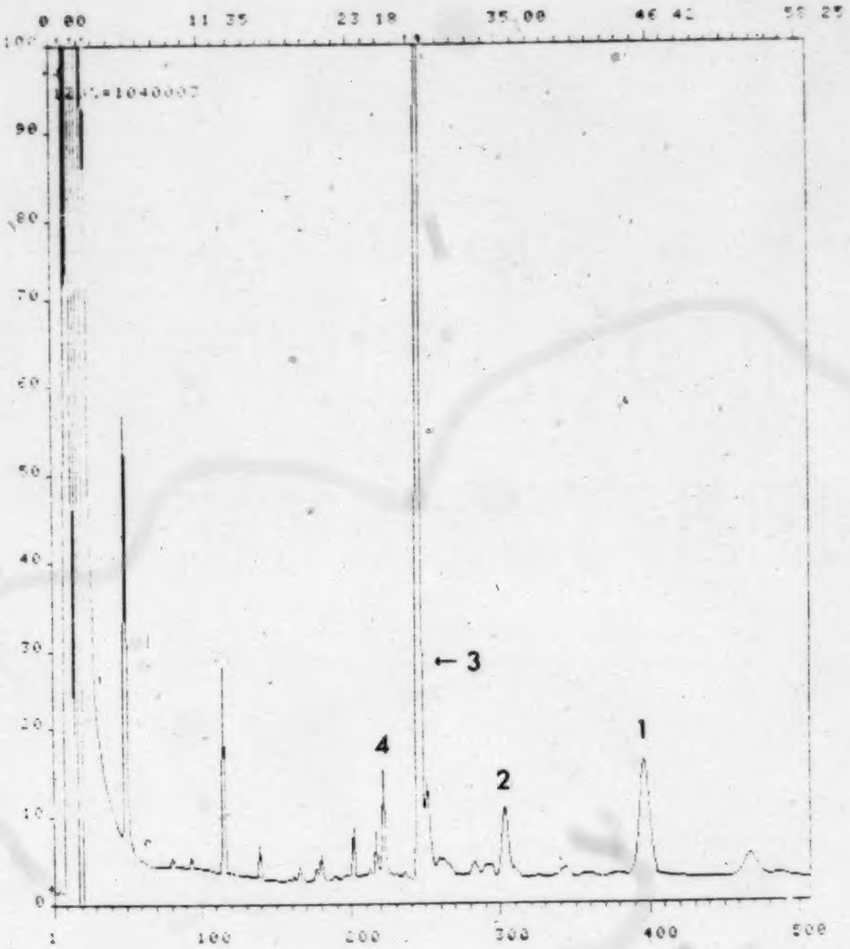

Figure 2. Chromatogram of A-methylester.

was identified by its mass spectral fragmentation pattern as listed in Table IV.

The next fraction investigated contained mainly component **B** (R_f 0.22, Skellysolve B/ethyl acetate 80:20) as shown by TLC over silica gel. Component **B** was purified by recrystallization from chloroform (amorphous white powder, mp = 238-242°).

In the HRMS* of **B** the highest mass ion is located at m/z 412 indicating a molecular formula of $C_{28}H_{44}O_2$. A peak at m/z 413 in the chemical ionization mass spectrum (CIMS, NH_3) of **B** confirms 412 as the probable molecular weight.

A strong hydroxyl absorption at 3400 cm^{-1} in the IR spectrum of **B**, combined with the absence of carbonyl stretching bands in the same, suggests that the two oxygens are present as alcohols.

A survey of the literature on naturally occurring compounds[38] and fungal metabolites[39] yielded only one compound, Portensterol (**1**), which matched the molecular formula ($C_{28}H_{44}O_2$).

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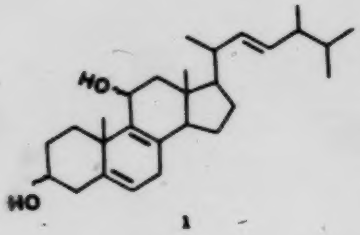
*The compositions of all fragment and parent ions reported in this work were determined by HRMS. Because of the small quantities involved, elemental analyses were not performed.

Table IV. Major fatty acids contained in **A**.

Peak	m/z (ester)	Formula (ester)	Formula (acid)	Name
1	354	$C_{23}H_{46}O_2$	$C_{22}H_{44}O_2$	Docasanoic
2	326	$C_{21}H_{42}O_2$	$C_{20}H_{40}O_2$	Eicosanoic
3	297	$C_{19}H_{37}O_2$	$C_{18}H_{34}O_2$	Octadecenoic
4	270	$C_{17}H_{34}O_2$	$C_{16}H_{32}O_2$	Hexadecanoic



1

Portensterol (**1**) is a sterol isolated from fruit bodies of Tricholoma portentosum, Rhodopaxillus nudus and Clitocybe nebularis.[40] It has a melting point of 250° (ethanol) and very similar IR and HRMS spectra to those of compound **B**. However, Portensterol (**1**) is reported as having poor solubility in organic solvents[40] while **B** is soluble in chloroform, acetone, and ethyl acetate. Furthermore, it has been reported that in the ^1H NMR of **1** the three vinylic protons appear as a multiplet at $\delta 5.25$;[40] the ^1H NMR of compound B, however, shows one proton signals at $\delta 5.19$ (dd, 8, 15 Hz),* $\delta 5.21$ (dd, 8, 19 Hz), and $\delta 5.38$ (bd, 5 Hz) (Figure 3). This strongly suggests that portensterol (**1**) and **B** are not the same compound.

It is possible that the ion at m/z 412 ($C_{28}H_{44}O_2$) in the HRMS of **B** does not correspond to the molecular ion but to a fragment resulting from the loss of the elements of water, a common process in steroidal alcohols and their

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*All NMR shifts are relative to tetramethylsilane.

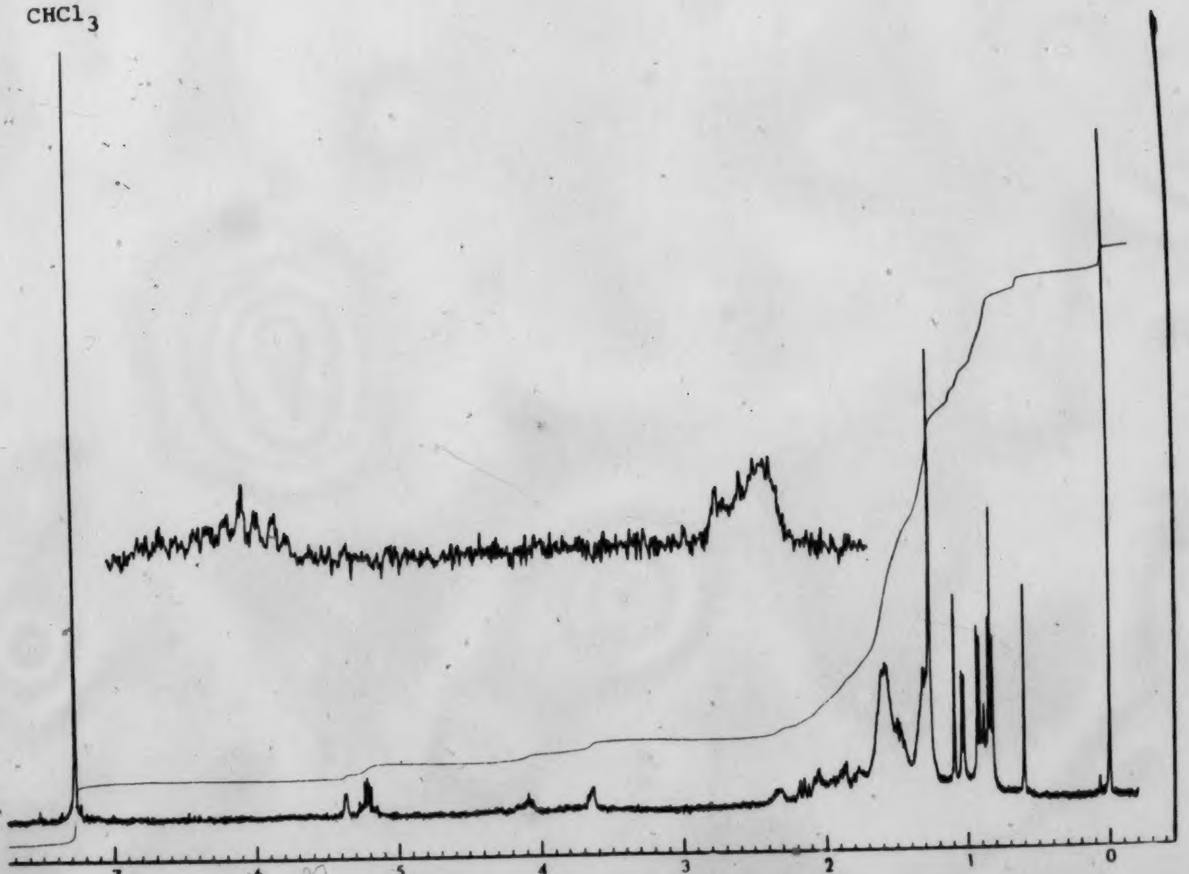
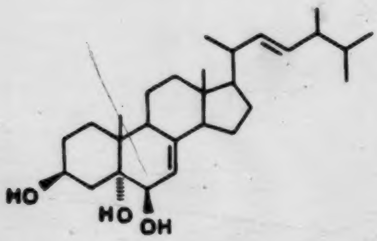


Figure 3. 400 MHz ^1H NMR spectrum ($C\underline{D}Cl_3$) of cerevisterol (**2**).

acetates.[41-43] If this is the case, the molecular weight of **B** is 430 and the molecular formula is $C_{28}H_{46}O_3$. This, together with other spectral data suggest that **B** is cerevisterol (**2**), a sterol previously isolated from a number of other fungi.[38,39]



2

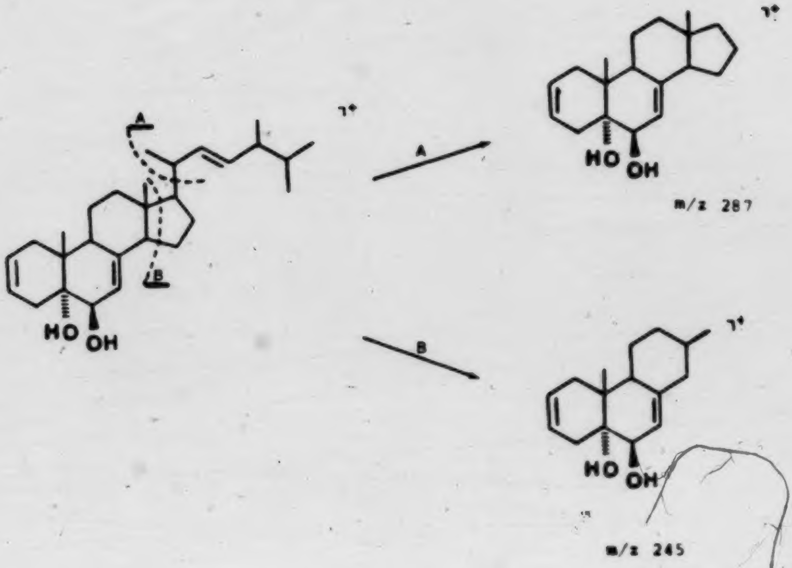
Cerevisterol (**2**) has a melting point of 252-255°,[44] and its reported IR and HRMS data[44,45] are very similar to that of compound **B**. Thus **B** was tentatively identified as **2**.

Several types of fragmentation reported for the mass spectra of steroids,[41-43], and which greatly aided in the identification of **B** as cerevisterol (**2**) are listed below:

1) The most common feature in the mass spectra of C-17 substituted steroids is the elimination of the side chain (path A, Scheme II) as well as the side chain plus 42 mass units (path B, Scheme II). This type of

Scheme II

MS Fragmentation of C-17 Substituted Steroids



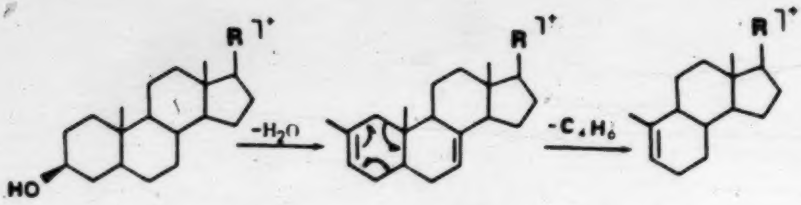
fragmentation provides sufficient information to determine the size of the side chain. In the case of compound B the following fragments originating from process A (loss of C_9H_{17}) were observed: m/z 287 ($C_{19}H_{27}O_2$, 7%), m/z 269 (287-H_2O, 30%) and m/z 251 (269-H_2O, 46%). Loss of [C_9H_{17} + 42] gives rise to fragments at m/z 245 ($C_{16}H_{21}O_2$, 3%), m/z 227 (245-H_2O, 10%) and 109 (227-H_2O, 6%). Each of these ions is reported in the MS of cerevisterol (**2**).[44]

2) The loss of ring A is a typical fragmentation reaction of steroidal hydrocarbons substituted at C-3 with

an hydroxyl group. The process involves initial elimination of water followed by decomposition of the ionized olefin *via* a retro Diels-Alder process (Scheme III). Compound **B** did not show the expected fragment at

Scheme III

Loss of Ring A in the MS of 3-Hydroxylated Steroids

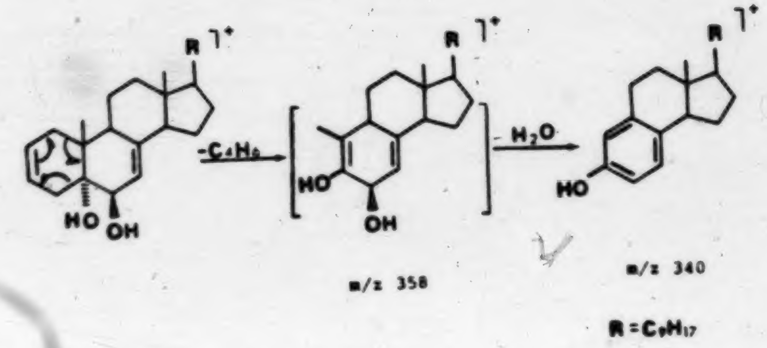


m/z 358 ($C_{24}H_{38}O_2$), instead a fragment at m/z 340 ($C_{24}H_{36}O$, 4%) was detected. This latter fragment results from loss of water leading to aromatization of ring B (Scheme IV).

(3) Cleavage of the 8-14 and 9-11 carbon to carbon bonds of the skeletal structure leaves rings A and B intact (Scheme V). This type of cleavage in compound **B** yields fragments at m/z 177 ($C_{11}H_{13}O_2$, 2%) and m/z 159 ($C_{11}H_{11}O$, 8%). Both fragments are reported in the HRMS of cerevisterol.[44]

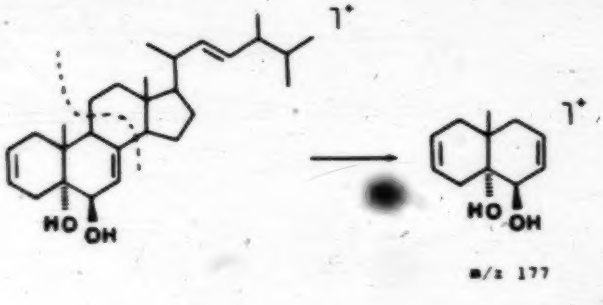
Scheme IV

Loss of Ring A in the MS of Cerevisterol


R
HO
OH
$-C_4H_6$
$-H_2O$
m/z 358
m/z 340
R = C_9H_{17}

Scheme V

Ring C Cleavage in the MS of Cerevisterol



Further evidence that **B** is cerevisterol (**2**) was evident from the ^1H NMR of **B**, which shows two one-proton signals attributable to hydrogens geminal to oxygen (δ 3.64 and δ 4.08), two three-proton singlets (δ 0.61 and δ 1.10), and four three-proton doublets (δ 0.85, 0.85, 0.93, 1.02; J = 7 Hz each).

Final confirmation that **B** is cerevisterol (**2**) was obtained by comparison of **B** with an authentic sample of cerevisterol.[46] Cerevisterol and **B** show identical TLC behavior and superimposable IR and ^1H NMR spectra.[46]

The third and most polar fraction (R_f 0.09, chloroform-methanol 95:5) was subjected to flash column chromatography[47] followed by recrystallization (ethyl-ether-methanol) to give component **C** as a white amorphous solid (mp 271-275°).

The identification of **C** presented several problems. Foremost among them was the fact that the number of hydrogens in the molecular formula, $C_{29}H_{50}O$ (mol. wt. 414) as determined by HRMS did not correspond to the number obtained by integration of the signals in the ^1H NMR spectrum (60 protons). Furthermore, the presence in the ^1H NMR of **C** (Figure 4) of eight signals (δ 4.42, d, 4 Hz; 3.87, dd, 2, 7 Hz; 3.77, dd, 2, 7 Hz; 3.66, bs; 3.61, m; 3.50, bd, 5 Hz; 3.40, m; 3.31, m) characteristic for hydrogens attached to carbons bearing oxygen could not be explained by the single oxygen in the formula.

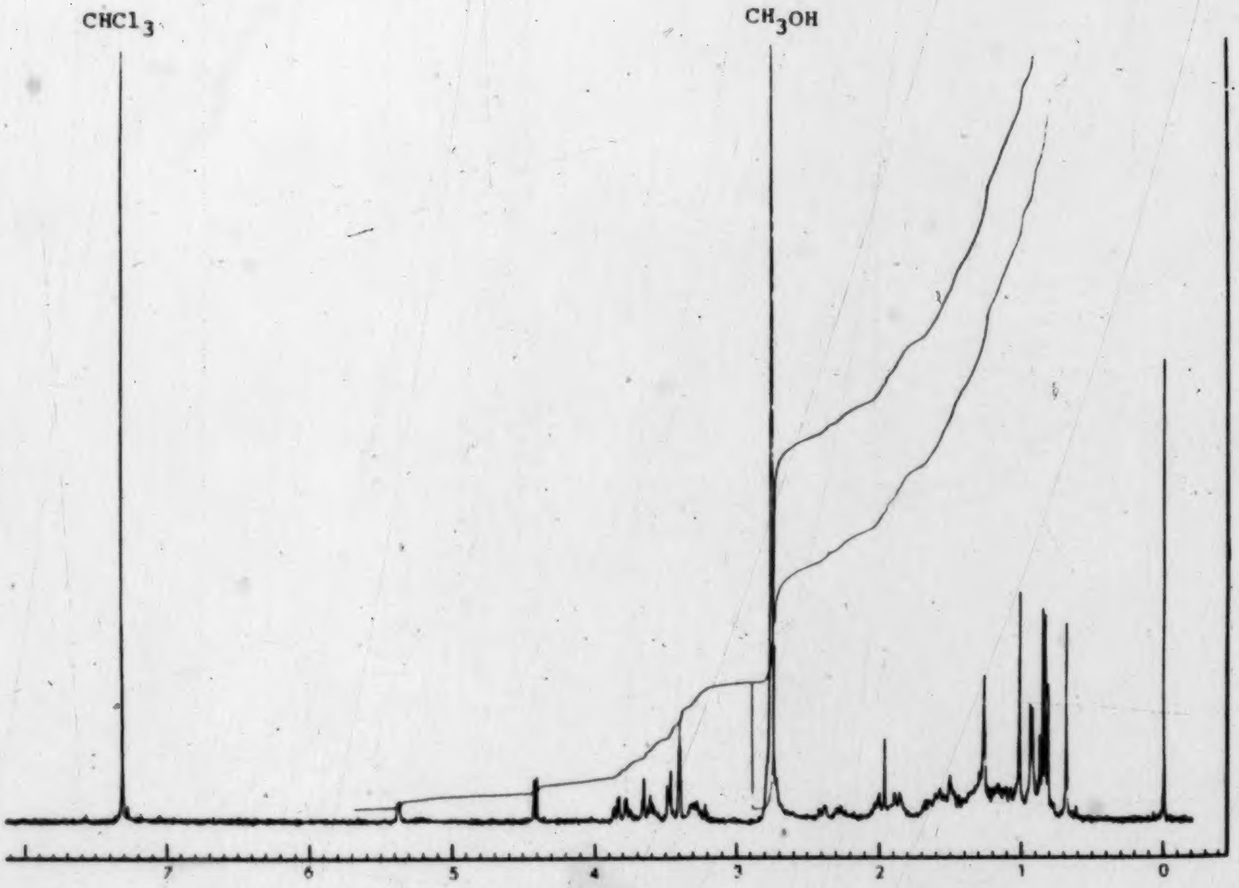


Figure 4. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3/C\underline{D}_3O\underline{D}$) of β-sitosteryl-β-D-glucoside (6).

Acetylation[*] of **C** resulted in the formation of a tetraacetylated derivative, **C'**, as indicated by methyl group singlets at δ2.08, 2.05, 2.02 and 2.00 in the ^{1}H NMR of **C'** (Figure 5). Furthermore, five of the eight signals in the ^{1}H NMR of **C** assigned to hydrogens geminal to oxygen experienced a significant downfield shift (δ5.22, dd, 8, 8 Hz; 5.08, dd, 8, 8 Hz; 4.96, dd, 8, 8 Hz; 4.11 and 4.26, ABX system, 12, 5, 2 Hz) upon formation of the tetraacetate **C'**. This provided enough evidence to conclude that of the four acetates, three were secondary and one originated from a primary alcohol.

The absence of hydroxyl absorption in the IR spectrum of **C'** proved the absence of tertiary alcohols. This suggested that the oxygens geminal to the unshifted proton signals (δ4.60, d, 8 Hz; 3.68, bs; 3.50, bs) in the ^{1}H NMR of **C'** are ethereal oxygens.

The HRMS of the tetraacetate **C'** is very similar to that of the alcohol **C**. However, the molecular weight of **C'** was determined to be 744 by the observation of a peak at m/z 762 in its CIMS (NH_3), corresponding to a complex of the tetraacetate **C'** and an ammonium ion. These results suggested that the compound could be a steroidal

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[*] Unless specified, all acetylation reactions were carried out using acetic anhydride-pyridine in a ratio 2:1.

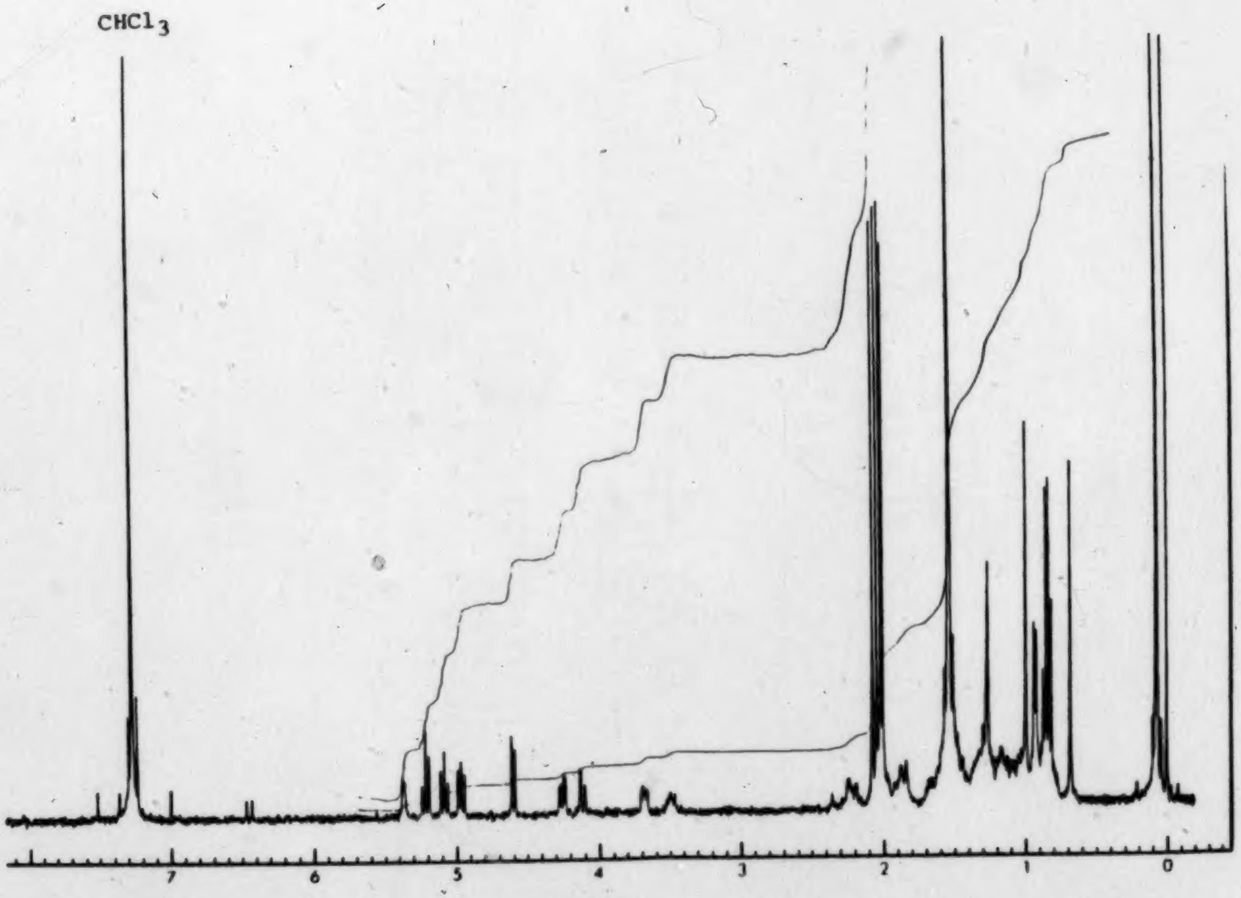
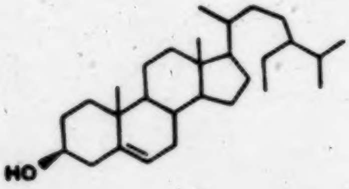


Figure 5. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of O,O,O,O-tetraacetyl-β-sitosteryl-β-D-glucoside (C').

glycoside.

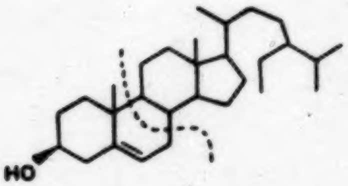
The steroidal component of **C** was tentatively identified as β-sitosterol (**3**) by its characteristic



3

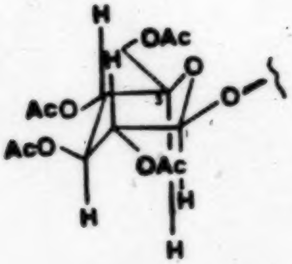
fragmentation pattern in the HRMS.[41-43,48] Compound **C**, like β-sitosterol (**3**), shows a strong peak at m/z 396 ($C_{29}H_{48}$, 100%) corresponding to the loss of the C-3 oxygen function. The expected M^+-111 fragment, characteristic of steroids having a C-5 - C-6 unsaturation,[41] is observed in the HRMS of **C** at m/z 303 ($C_{22}H_{39}$, 4%). Loss of the C-17 side chain and the side chain plus 42 mass units (see Scheme II) gives rise to fragments at m/3 273 ($C_{19}H_{29}O$, 2%), m/z 255 ($C_{19}H_{27}$, 12%), m/z 231 ($C_{16}H_{23}O$, 2%) and m/z 213 ($C_{16}H_{21}$, 9%) in the HRMS of **C**. These fragment ions are also reported in the case of β-sitosterol.[41,48] Cleavage of the molecule at the 9-10 and 7-8 carbon to carbon bonds (**4**)[41] together with the elimination of water gives fragments at m/z 119 (C_9H_{11}, 10%), and m/z 287 ($C_{21}H_{35}$, 1%) in the HRMS of **C**. These peaks are also observed for β-sitosterol (**3**).[41,48]

Since the tetraacetate gave a better resolved ^{1}H NMR (Figure 5) spectrum this was used in determining the



4

nature of the sugar moiety. The proton at C-1 appears as a doublet ($J_{1,2}$ = 8 Hz)* with a chemical shift of 4.60. The signal for H-2 is a doublet of doublets at δ 4.96 ($J_{2,1}$ = 8 Hz, $J_{2,3}$ = 8 Hz) as are the signals for H-3 and H-4 at δ 5.22 and 5.08. This can only be explained by a glucoside structure where all the protons are in the axial orientation (see 5). Furthermore, the chemical shifts



5

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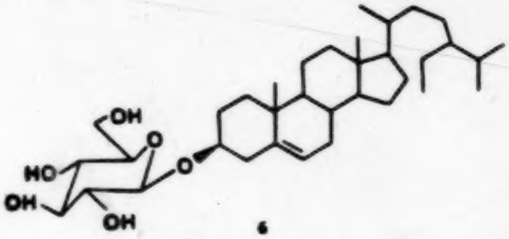
*Verified by decoupling experiments.

observed for H-3 and H-5 (δ 3.68 and δ3.50 respectively) are in agreement with the values reported for β-D-glycosides.[49] In the anomeric case (i.e. α-D) the H-2 signal appears as a quartet ($J_{2,1}$ = 3.5 Hz and $J_{2,3}$ = 10 Hz) while the triplet for H-3 is shifted downfield due to the 1,3-diaxial deshielding effect of the glycoside bond.[49] This and the reported data[48,49] are in agreement with the identification of **C** as β-sitosteryl-β-d-glucoside (**6**).



6

As mentioned, the metabolites from the mycelium did not show activity against canola. By this time, however, a reliable reproducible bioassay* had been developed, similar to that used in the isolation of the AM-toxins (6a-6c)[11] from Alternaria mali (apple pathotype of Alternaria alternata, see Table I), the fungus reported to be the cause of alternaria blotch of apple.[9] In this bioassay the test component is absorbed on silica gel.

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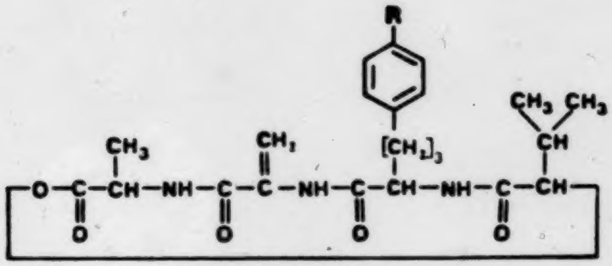
*For a complete description see Experimental.



6a AM-toxin I R = $-OCH_3$
6b AM-toxin II R = H
6c AM-toxin III R = OH

The impregnated silica gel is placed on the canola leaf and wetted with a small amount of water. The leaf is placed in a petri dish in a moist atmosphere at room temperature. In a positive test, chlorosis is usually observed within 48 hours. Using this assay procedure it was possible to determine that strong biological activity was present only in the organic extracts of the broth from A. brassicae grown in V-8 juice medium. It is not detected in the organic extracts from other culture media. As a result of this observation, the growth of A. brassicae in the other media was discontinued.

Purification of the active crude extract was initiated by redissolving it in a mixture of water-methanol (ca. 9:1, v/v), and successively partitioning the resulting suspension between hexane (low-polarity fraction), ethyl acetate (medium-polarity fraction), and n-butanol (high-polarity fraction). Bioassay of the three fractions showed that the medium polarity fraction, which was the largest fraction, possessed the strongest chlorosis-inducing ability. Further separation of the medium polarity fraction by "dry flash" column chromatography using gradient elution (Skellysolve B-Acetone) gave 18 major fractions.

"Dry flash" column chromatography is a precedure developed during this work* to eliminate the loading problems encountered when the sample to be purified is not readily soluble in the eluant mixture. Selection and initial packing of the column is done according to Still's procedure.[47]

The sample is adsorbed on silica gel and then placed as a thin, even band at the top of the column. It is then covered with _ca._ 2" of sand. Expulsion of the air and final packing of the column is accomplished by allowing the least polar solvent of the eluant mixture to pass through the column. At this point the flow rate is also regulated, and separation is initiated by adding the desired eluant. The remainder of the chromatographic procedure is similar to that of normal flash chromatography.[47]

Each of the 18 fractions obtained by separation of the medium polarity extract was tested for biological activity. Fractions 10 and 11 gave positive results, both fractions showing the same major component by TLC[*] (R_f 0.35, benzene-acetone 70:30).

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*For complete description see Experimental.

Further purification of these two combined fractions by "dry flash" column chromatography (benzene-acetone 80:20) yielded two pure compounds which differed in the way they visualize on TLC. The minor component, **D**, appears with a characteristic blue color; the most abundant, crystalline component **E** is barely visible.[**]

Component **E** was purified by recrystallization from Skellysolve B-acetone to give optically active white needles (mp 225-227°) and was found to have a molecular formula of $C_{30}H_{51}N_5O_7$ (mol. wt. 593) from its HRMS. The molecular weight of **E** was confirmed by fast atom bombardment (FAB) mass spectrometry where peaks at m/z 1188 [2(M+1)] and m/z 594 (M+1) are observed. The molecular formula of **E** suggests that it may be a polypeptide. Further evidence for the peptide nature of **E** was given by the similarity of its IR spectrum (Figure 6)

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[*] Unless specified, for the remaining of Part I of this work the visualization method used involves spraying the TLC plate with phosphomolibdic acid reagent (see appendix of Experimental for composition) followed by charring.

[**] A better way to visualize it is by first placing the plate under I_2 vapors, spraying with a benzoflavone solution (0.3 g of 7,8-benzoflavone in 95 mL of ethanol and 5 mL of 30% H_2SO_4) and finally spraying with the phosphomolybdic acid reagent followed by charring.

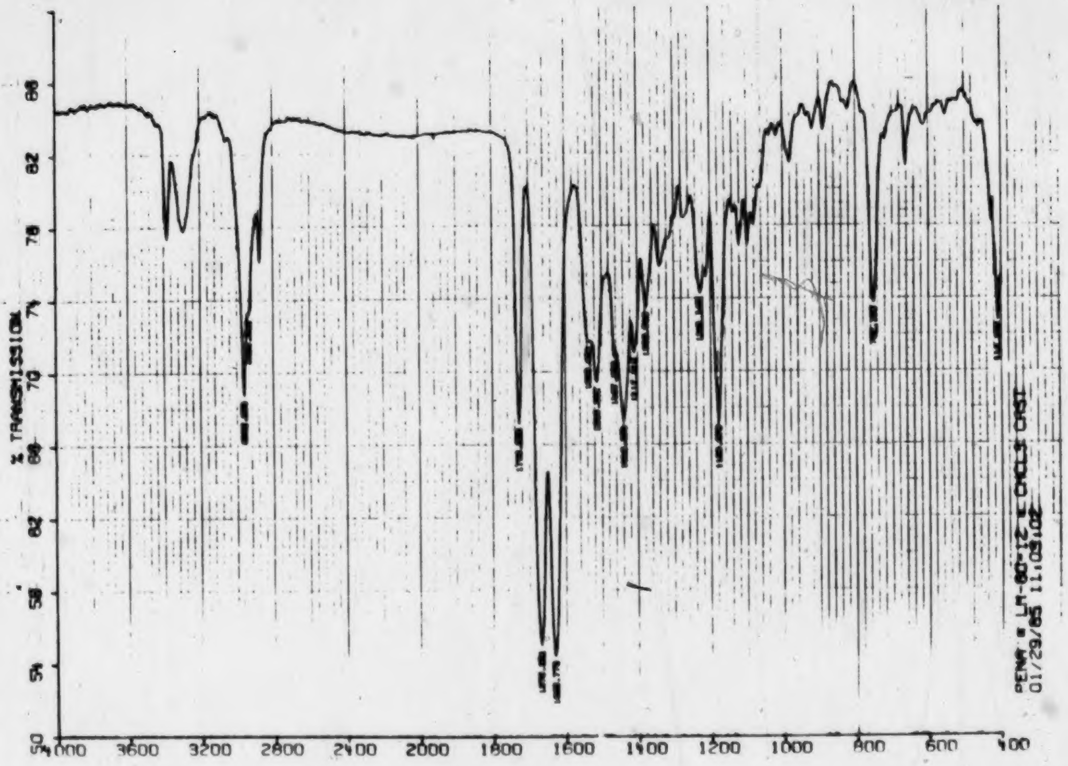


Figure 6. IR spectrum ($CHCl_3$ cast) of destruxin B (13).

with that reported for cyclodepsipeptides[50] including the AM-toxins (6a-6c).

Cyclodepsipeptides are heteromeric peptides in which a macrocyclic ring is formed from amino- and hydroxy acids connected through amide and ester bonds. These compounds show characteristic IR absorption.[50] The IR spectrum of **E** (Figure 6) shows this characteristic spectrum displaying N-H bands at 3360 cm^{-1} and 3290 cm^{-1}, an ester carbonyl absorption at 1730 cm^{-1}, two amide I bands at 1670 cm^{-1} and 1630 cm^{-1}, and an amide II band at 1515 cm^{-1}.

The seven oxygens of compound **E** may be accounted for as follows: five as amide carbonyls and two as ester oxygens. If **E** is a cyclodepsipeptide then seven of the eight unsaturations implied by the molecular formula are accounted for as well. The final unsaturation must be a second ring since no vinylic proton signals are observed in the ^1H NMR of compound **E**, and no olefinic sp^2 carbons are observed in the ^{13}C NMR of **E**.

The ^1H NMR of compound **E** (Figure 7) shows some striking similarities to that of tentoxin (**7**), a metabolite of Alternaria tenuis,[27-30] the phytopathogen which causes chlorosis in several different plant species. The similar signals include two doublets at $\delta 8.19$ and $\delta 7.17$ ($J = 9$ Hz each) corresponding to hydrogens on nitrogen, and two three-proton sharp singlets at $\delta 3.20$

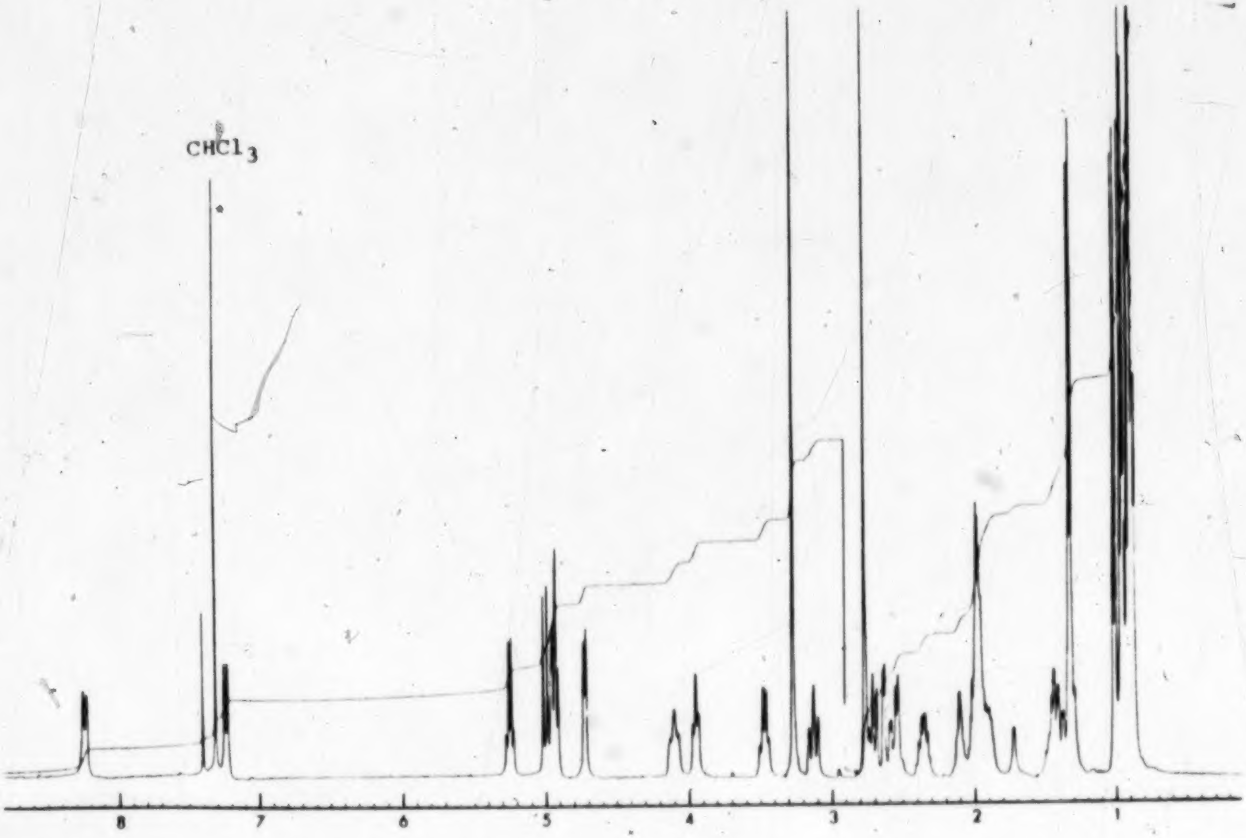
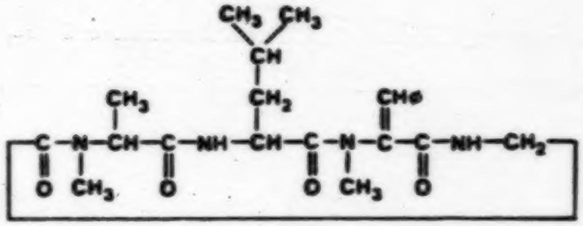


Figure 7. 400 MHz ^{1}H NMR spectrum ($C\underline{D}Cl_3$) of destruxin B (**13**).



7

and δ2.73 characteristic of methyl groups attached to nitrogen. These data allow us to account for four of the nitrogens of the molecular formula of **E**, consequently the remaining nitrogen must be tertiary.

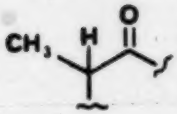
Further analysis of the ^{1}H NMR of compound **E** indicates the presence of seven more methyl groups (total = 9), all of them on carbon, six as doublets (J = 6-7 Hz each) and one as a triplet. Table V lists the protons, with their corresponding chemical shifts, of compound **E**. Table VI shows the spin systems confirmed by spin-spin decoupling experiments.

The isolated methyl doublet at δ1.30 (J = 7 Hz) was assigned to fragment **i**. One proton on nitrogen (δ8.19, d, 9 Hz) is coupled to the methylene signals at δ4.05 (ddd, 13.5, 10, 5, 2 Hz) and δ3.08 (dddd, 13.9, 11.5, 2, 1.5 Hz), each of which show further coupling to two other protons (δ2.66 and δ2.59) indicating the presence of a

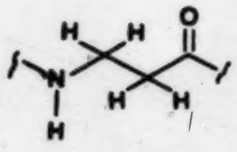
Table V. Chemical shifts of the protons of compound **E**.

Proton Number	Chemical Shift	
1	8.19	d, 9 Hz; 1H
2	7.17	d, 9 Hz; 1H
3	5.19	q, 6.5 Hz; 1H
4	4.94	d, 11 Hz; 1H
5	4.84	dd, 3, 10 Hz; 1H
6	4.83	dd, 9, 6.5 Hz; 1H
7	4.67	bd, 7 Hz; 1H
8	4.05	dddd, 13.5, 10, 5, 2 Hz; 1H
9	3.91	bt, 9 Hz; 1H
10	3.43	bq, 9 Hz; 1H
11	3.20	s; 3H
12	3.08	dddd, 13.5, 11.5, 2, 1.5 Hz; 1H
13	2.73	s; 3H
14	2.66	ddd, 18, 11.5, 2 Hz; 1H
15	2.59	ddd, 18, 5, 1.5 Hz; 1H
16	2.49	m; 1H
17	2.32	dsept, 11, 6.5 Hz; 1H
18	2.08	bs; 1H
19	1.90	m; 7H
20	1.30	d, 7 Hz; 3H
21	0.99	d, 6.5 Hz; 3H
22	0.94	d, 6.0 Hz; 3H
23	0.93	d, 6.0 Hz; 3H
24	0.89	d, 6.5 Hz; 3H
25	0.86	d, 7.0 Hz; 3H
26	0.86	t, 7.5 Hz; 3H

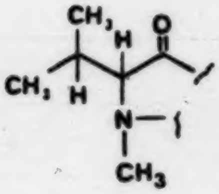
Table VI. Spin systems of compound **E**.



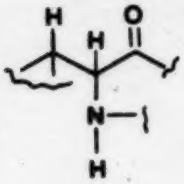
i



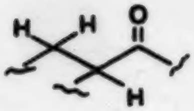
ii



iii



iv



v

neighboring methylene group. This system was assigned as fragment **ii.** A one proton signal at δ2.32 (d sept, 11, 6.5 Hz) corresponding to the methine proton of an isopropyl group is coupled to a proton attached to a carbon bearing an N-methyl group (sharp doublet, J = 11 Hz, δ4.94). This spin system was assigned as fragment **iii.** The one-proton doublet of doublets at δ4.84 (J = 3, 10 Hz) coupled to a second proton on nitrogen (δ7.17, d, 9 Hz) and to a methine hydrogen was assigned to fragment **iv.**

A second doublet of doublets at δ4.83 (J = 9, 6.5 Hz) is coupled to only a methylene group as shown for fragment **v.**

These ^{1}H NMR data were complemented when compound **E** was subjected to acid hydrolysis by treatment with 6N hydrochloric acid in a sealed tube at 100°C for 24 h. The amino acids produced were determined by an amino acid autoanalyzer[*] as β-alanine (**8**), isoleucine (**9**), and proline (**10**), present in equimolar quantities. On the basis of the chemical shifts and coupling patterns observed fragment **ii** can be incorporated into the structure of β-alanine (**8**). Incorporation of fragment **iv** into the structure of isoleucine (**9**)results in the new

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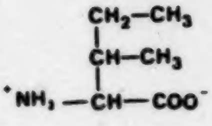
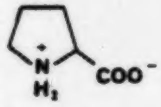
[*] Analysis performed by Mr. M. Natriss of the Department of Biochemistry.

$^{+}NH_3-CH_2-CH_2-COO^{-}$

8

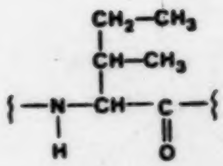


9



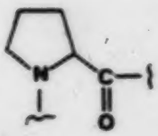
10

fragment **vi**, all the proton signals for which are present in the ^{1}H NMR of **E**.



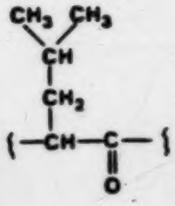
vi

One methine (δ 4.67) and two methylene protons (δ 3.91 and δ 3.43) on carbons α to nitrogen fit very well in a proline-type of structure as shown in fragment **vii**. The proline moiety present in **E** accounts for the eighth unsaturation implied by the molecular formula of compound **E**.



vii

Table VII displays the C-13 values and multiplicity of all the carbons in compound **E** (Figure 8). They are divided as follows: six quaternary carbons, eight methines, seven methylenes and nine methyl groups. Fragments **i**, **ii**, **iii**, **v**, **vi**, and **vii** account for all the carbons except an isopropyl group. This can be placed in fragment **v**, the only one incomplete, to give the new fragment **viii**.



viii

What remains to be determined is the location of the second N-methyl group since it can be part of fragments **i** or **vii**, and sufficient information for the unequivocal assignment is not readily available from the ^1H NMR of **E**. However, the problem can be solved from the knowledge of the order of the amino acids in the cyclodepsipeptide. This can be determined by using either chemical methods, which are slow and cumbersome, or with the aid of HRMS.

It has been reported that sequence analysis of cyclodepsipeptides by HRMS may give inconclusive results

Table VII. C-13 values of carbons in compound **E**.

173.70 (s)
173.55 (s)
171.08 (s)
170.93 (s)
*169.66 (s)
71.95 (d)
60.71 (d)
58.13 (d)
55.46 (d)
53.66 (d)

46.44 (t)
38.95 (t)
37.53 (d)
34.42 (t)
33.21 (t)
30.80 (d)
28.85 (t)
28.03 (d)
27.23 (q)
24.49 (t)

24.41 (q)
24.10 (t)
24.32 (q)
21.49 (q)
20.00 (q)
19.62 (q)
15.37 (q)
15.21 (q)
11.32 (q)

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*Two carbons.

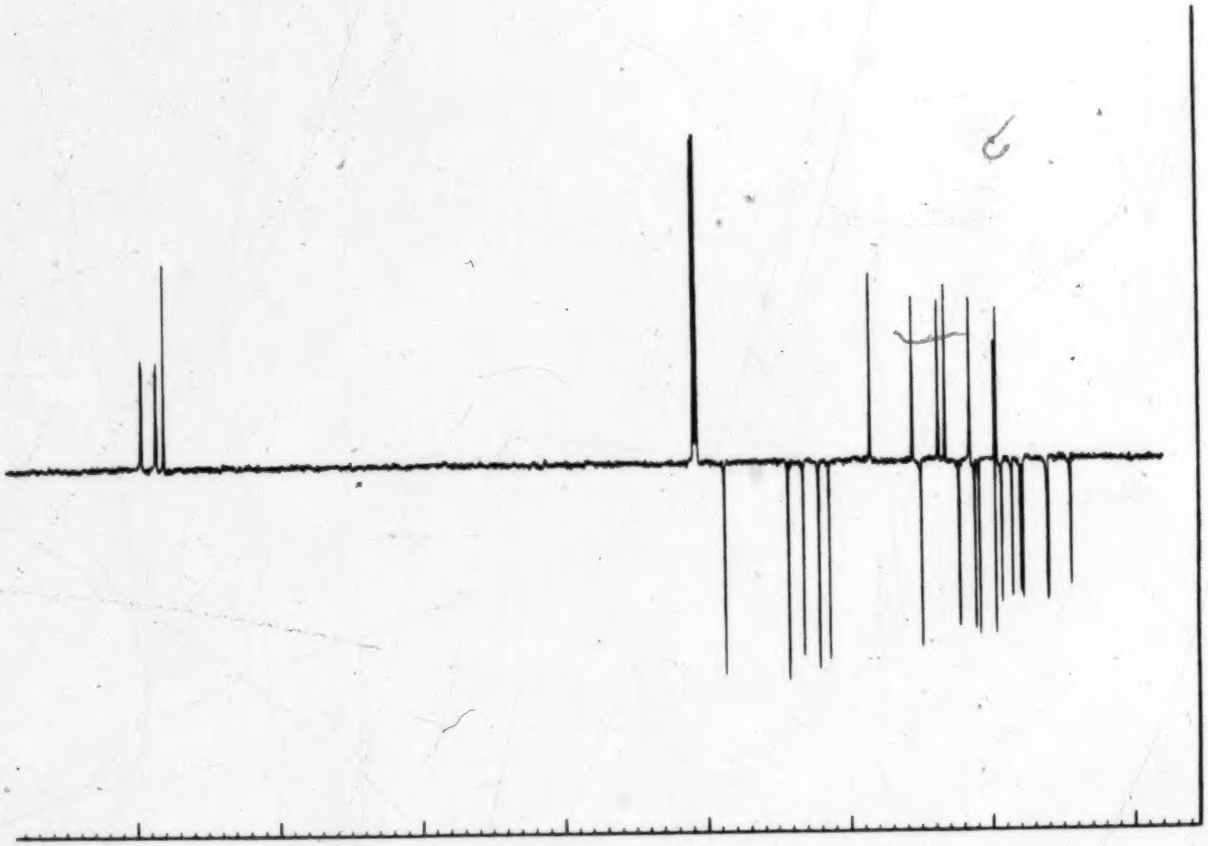


Figure 8. APT ^{13}C NMR spectrum ($C\underline{D}Cl_3$) of destruxin B (**13**).

due to the rearrangement and recombination of ions.[11,51] On the other hand sequence analysis of the acyclic hydroxymethyl ester derivative obtained by methanolysis of the cyclodepsipeptide allows determination of the amino acid sequence with reasonable certainty.[11,20,21]

The hydroxymethyl ester, compound **E'**, was prepared by stirring a methanolic solution of **E** with a catalytic amount of potassium carbonate for 12 h. Chromatographic purification of the reaction product afforded **E'** as an oil. The HRMS of **E'** shows a parent peak at m/z 625 ($C_{31}H_{55}N_5O_7$) as expected. Its ^1H NMR showed that **E'**, which by TLC appeared to be a single compound, is a mixture of two components present in a ratio of 2:1. The fact that it is a mixture was indicated by the appearance of four N-H signals at δ 7.68, 7.41, 6.99 and 6.47; two methoxyl singlets at δ 3.62 and 3.61; four N-methyl singlets at δ 3.19, 3.00, 2.95 and 2.71, and two methyl doublets at δ 1.29 and 1.21.

The mixture could not be separated using a variety of chromatographic techniques. In an attempt to simplify the purification, the methanolysis product **E'** was acetylated to yield compound **E"**. The TLC of **E"** showed only one spot in several solvent systems. In the HRMS of **E"** the expected parent peak at m/z 667 ($C_{33}H_{57}N_5O_9$) is observed. The ^1H NMR of **E"** shows two signals corresponding to acetyl methyls (δ 2.12 and 2.14) indicating that **E"** is indeed a mixture of two compounds. Attempted chromatographic separation of the mixture proved unsuccessful. The explanation for the difficulty in separating this mixture is presumably that the two products are diastereomers, formed by epimerization at one or more of the chiral centers. Previous workers have reported that diastereomers of depsipeptides are difficult to separate.[50] Nevertheless, the sequence analysis results are useful since the data resulting from the mass spectrometric analysis of compounds **E'** and **E"** are not affected by the presence of diastereomers.

It has been reported that the MS fragmentation of a linear polypeptide proceeds by the two major pathways[20,21,52] shown in Scheme VI. Of the two, the "amine fragmentation" (**a**) is the most important.

Scheme VI

Fragmentation Pattern in the MS of Linear Polypeptides

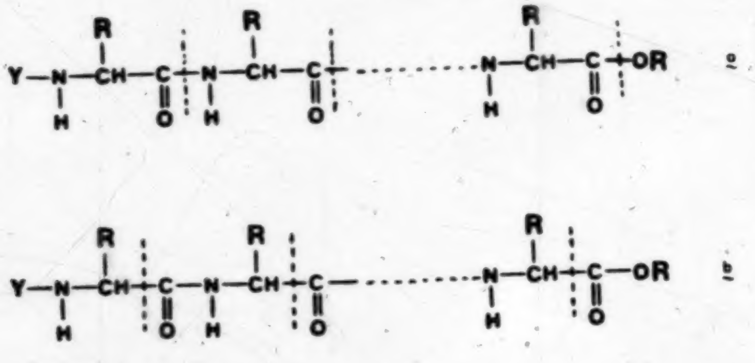
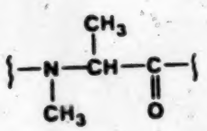
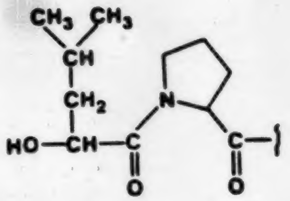


Table VIII shows the fragment ions observed in the HRMS of compounds **E'** and **E"**. After the initial loss of the methoxyl group ($M^+ -31$), the first fragment to be lost is that with the formula C_3H_6NO which was assigned to a protonated fragment **ii**. The second fragment lost is the one with the formula C_4H_6NO, which can only be explained if the unassigned N-methyl group is part of fragment **i** to give the new fragment **ix**. The next two fragmentations can be due to the loss of fragments **iii** or **vi**, both C-6, in either order. The remaining structure, having eleven carbons, must contain fragments **vii** and **viii** with the latter being the hydroxyacid moiety of **E**, as it is shown in **x**.



$\}-\underset{\underset{CH_3}{|}}{N}-\overset{\overset{CH_3}{|}}{CH}-\underset{\underset{O}{\|}}{C}-\{$

ix

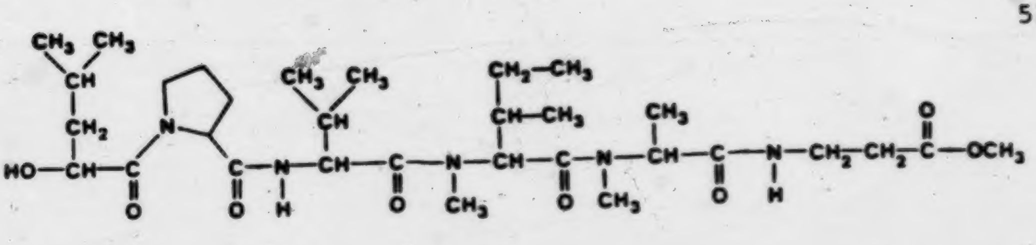


x

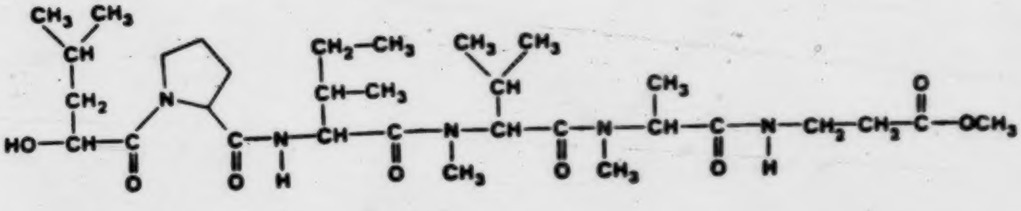
With all these data, the structure of **E'** can be proposed as either **11** or **12**. To determine the correct order of the aminoacids in compound **E**, i.e. that in structures **11** or **12**, it was necessary to analyze the HRMS of **E**; since it has been reported that there are a number of typical MS fragmentations for a cyclodepsipeptide having an N-methyl-valyl-N-methylalanyl moiety.[53] These are shown in Table

Table VIII. Fragment ions in the HRMS of **E'** and **E"**.

E' m/z	**E"** m/z	**E'** inf LM-80-12-OMe	**E"** inf LM-84
625	667	M^+	M^+
594	636	M^+-OCH_3	M^+-OCH_3
522	564	695-C_3H_6NO	636-C_3H_6NO
438	480	522-C_4H_6NO	564-C_4H_6NO
325	367	438-$C_6H_{11}NO$	480-$C_6H_{11}NO$
212	254	326-$C_6H_{12}NO$	368-$C_6H_{12}NO$
86	86	Base Peak	Base Peak



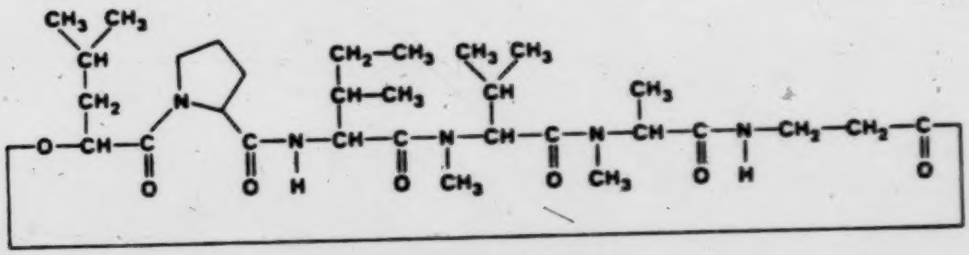
11



12

IX along with the fragment ions observed in the HRMS of **E**.

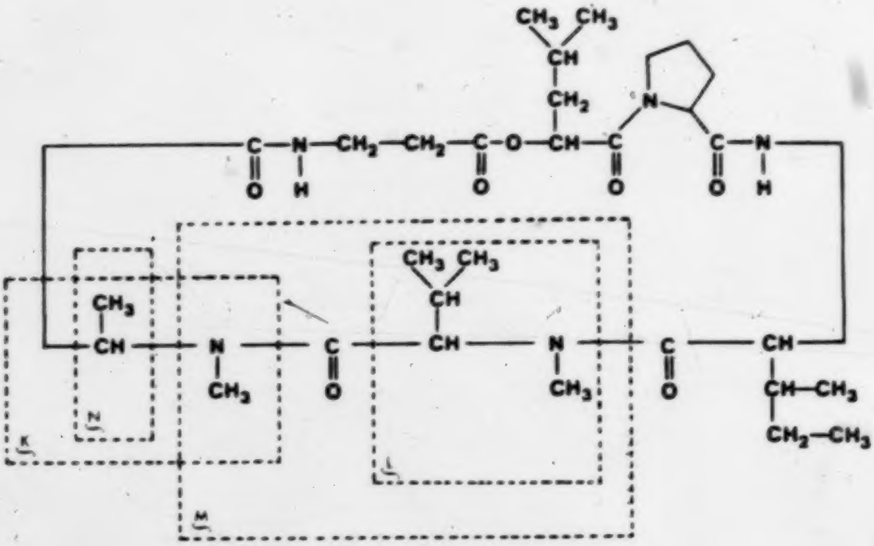
The results obtained indicate that the order of the aminoacids is that shown for **E'** in **12**, and consequently compound **E** has structure **13**.



13

Structure **13** is the structure reported for destruxin, B[32,33,39] an insecticidal metabolite isolated from the fungus Metarrhizium anisopliae (initially known as Oospora destructor[32]), a fungus pathogenic to silkworms.[54,55] Final confirmation was obtained by comparison of compound **E** from A. brassicae with an authentic sample of destruxin

Table IX. Typical MS fragmentations in a cyclodepsipeptide having an N-methylvalyl-N-methylalanyl moiety.



Destruxin B

m/z	Formula	inf
593	$C_{30}H_{51}N_5O_7$	M^+
536	$C_{27}H_{44}N_4O_7$	M^+-Fragment **K**
508	$C_{25}H_{40}N_4O_7$	M^+-Fragment **L**
451	$C_{23}H_{37}N_3O_6$	M^+-Fragment **M**
423	$C_{21}H_{33}N_3O_6$	451-Fragment **N**
86	$C_5H_{12}N$	Base Peak

B obtained from M. anisopliae.* These compounds were shown to have superimposable ^{1}H NMR and IR spectra, identical optical rotation, and gave an unsuppressed melting point (225-227°), thus **E** is destruxin B (**13**).

It has been suggested since destruxin B (**13**) has been found in silkworm larvae infected with Metarrhizium anisopliae, that destruxin B (**13**) plays an important role in the mycosis to kill the hosts. However, it is reported not to show appreciable antimicrobial activity.[54] In our case pure destruxin B (**13**) has been tested for chlorosis inducing ability in concentrations of 5, 2.5, and 1%. In every case strong biological activity was observed after 48 h. This confirms destruxin B (**13**) as one of, if not the only, chlorosis inducing component.

In addition to destruxin B (**13**) a series of related cyclodepsipeptides have been isolated and identified from M. anisopliae. Table X lists all of the destruxins reported to date.[33]

A second, less polar component, **F**, whose TLC charring behavior was similar to that of destruxin B (**13**) was present in the fractions of the original chromatographic separation. Further purification of a fraction containing

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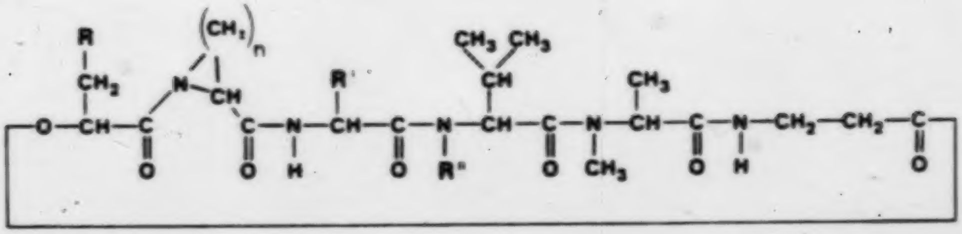
*Sample kindly supplied by Dr. M. Pais, Institut de Chimie des Substances Naturelles, C.N.R.S., 91190-Gif-Sur-Yvette, France.

Table X. Destruxins reported to date.

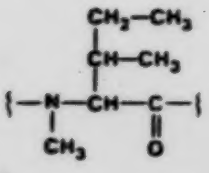


Name	R	n	R'	R"
Destruxin A	$-CH=CH_2$	3	$-CHMeCH_2Me$	Me
Destruxin B	$-CHMe_2$	3	$-CHMeCH_2Me$	Me
Destruxin C	$-CHMeCH_2OH$	3	$-CHMeCH_2Me$	Me
Destruxin D	$-CHMeCOOH$	3	$-CHMeCH_2Me$	Me
Desmethyldestruxin B	$-CHMe_2$	3	$-CHMeCH_2Me$	H
Destruxin E	$-\underset{\diagdown O \diagup}{CH - CH_2}$	3	$-CHMeCH_2Me$	Me
Destruxin A_1	$-CH=CH_2$	4	$-CHMeCH_2Me$	Me
Destruxin A_2	$-CH=CH_2$	3	$-CHMe_2$	Me
Destruxin B_1	$-CHMe_2$	4	$-CHMeCH_2Me$	Me
Destruxin B_2	$-CHMe_2$	3	$-CHMe_2$	Me
Destruxin C_2	$-CHMeCH_2OH$	3	$-CHMe_2$	Me
Destruxin D_1	$-CHMeCH_2OH$	4	$-CHMeCH_2Me$	Me
Destruxin D_2	$-CHMeCOOH$	3	$-CHMe_2$	Me
Destruxin E_1	$-\underset{\diagdown O \diagup}{CH - CH_2}$	4	$-CHMeCH_2Me$	Me

both destruxin B (**13**) and component **F**, by preparative TLC, afforded each in pure form.

The HRMS of **F** shows a molecular ion peak at m/z 607 corresponding to the formula $C_{31}H_{53}N_5O_7$. The molecular formula of **F** differs from that of destruxin B (**13**) by a methylene group and suggests that component **F** may be a second cyclodepsipeptide.

The name homodestruxin B was given to **F** to reflect the fact that it contains one more methylene equivalent than destruxin (**13**). In order to determine the location of the extra methylene in homodestruxin B the HRMS fragmentation pattern was compared to that observed for destruxin B (**13**). The fragment ions of each compound are listed in Table XI. Inspection of the data reveals that fragments **L** and **M** (see Table VIII) show an increase of 14 mass units. Consequently homodestruxin B does not contain an N-methylvaline (Fragment **L**, Table XI) moiety, instead either an N-methylisoleucine (**14**) or N-methylleucine (**15**) group is present.



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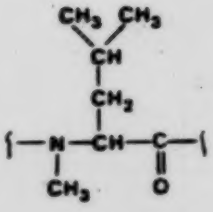


15

Table XI. MS fragmentation of destruxin and homodestruxin B.

Destruxin B			Homodestruxin B		
m/z	Formula	inf	m/z	Formula	inf
593	$C_{30}H_{51}N_5O_7$	M^+	607	$C_{31}H_{53}N_5O_7$	M^+
536	$C_{27}H_{44}N_4O_7$	$M^+-C_3H_7N$ (**K**)	550	$C_{28}H_{46}N_4O_7$	$M^+-C_3H_7N$
508	$C_{25}H_{40}N_4O_7$	$M^+-C_5H_{11}N$ (**L**)	508	$C_{25}H_{40}N_4O_7$	$M^+-C_6H_{13}N$
451	$C_{23}H_{37}N_3O_6$	$M^+-C_7H_{14}N_2O$ (**M**)	451	$C_{23}H_{37}N_3O_6$	$M^+-C_8H_{16}N_2O$
423	$C_{21}H_{33}N_3O_6$	$451-C_2H_4$ (**N**)	423	$C_{21}H_{33}N_3O_6$	$451-C_2H_4$
86		Base Peak	100	$C_6H_{14}N$	Base Peak

Final confirmation of the absence of the N-methylvaline unit in homodestruxin B is provided by its ^1H NMR spectrum. The ^1H NMR of destruxin B (**13**) is very similar to that of homodestruxin B (Figure 9) except that the doublet of septets at δ 2.23 (see signal 17, Table IV) is absent in the ^1H NMR of homodestruxin B. Furthermore, the ^1H NMR of homodestruxin B shows two methyl triplets (δ 0.88, t, 7 Hz; 0.81, t, 7 Hz) in place of the single one observed for destruxin B. A methine proton at δ 4.99, characteristic of hydrogens α to nitrogen, appears as a sharp doublet (J = 11 Hz) in the ^1H NMR of homodestruxin B. These data are consistant with an N-methylisoleucine (**14**) group in homodestruxin B.

The hydroxymethyl ester derivative of homodestruxin B was prepared and its HRMS was compared to that of the hydroxymethyl ester derivative of destruxin B (**12**) (Table XII). The loss of the N-methylvaline fragment (438 - $C_6H_{11}NO$) in the HRMS of the hydroxymethyl ester of destruxin B (**12**) is replaced by the loss of a fragment corresponding to **14** (452 - $C_7H_{13}NO$) in that of the hydroxymethyl ester derivative of homodestruxin B. The homodestruxin B can be assigned structure **16** in which the N-methylvaline unit of **13** has been replaced with N-methylisoleucine. The hydroxymethyl ester derivative of **16** thus has structure **17**.

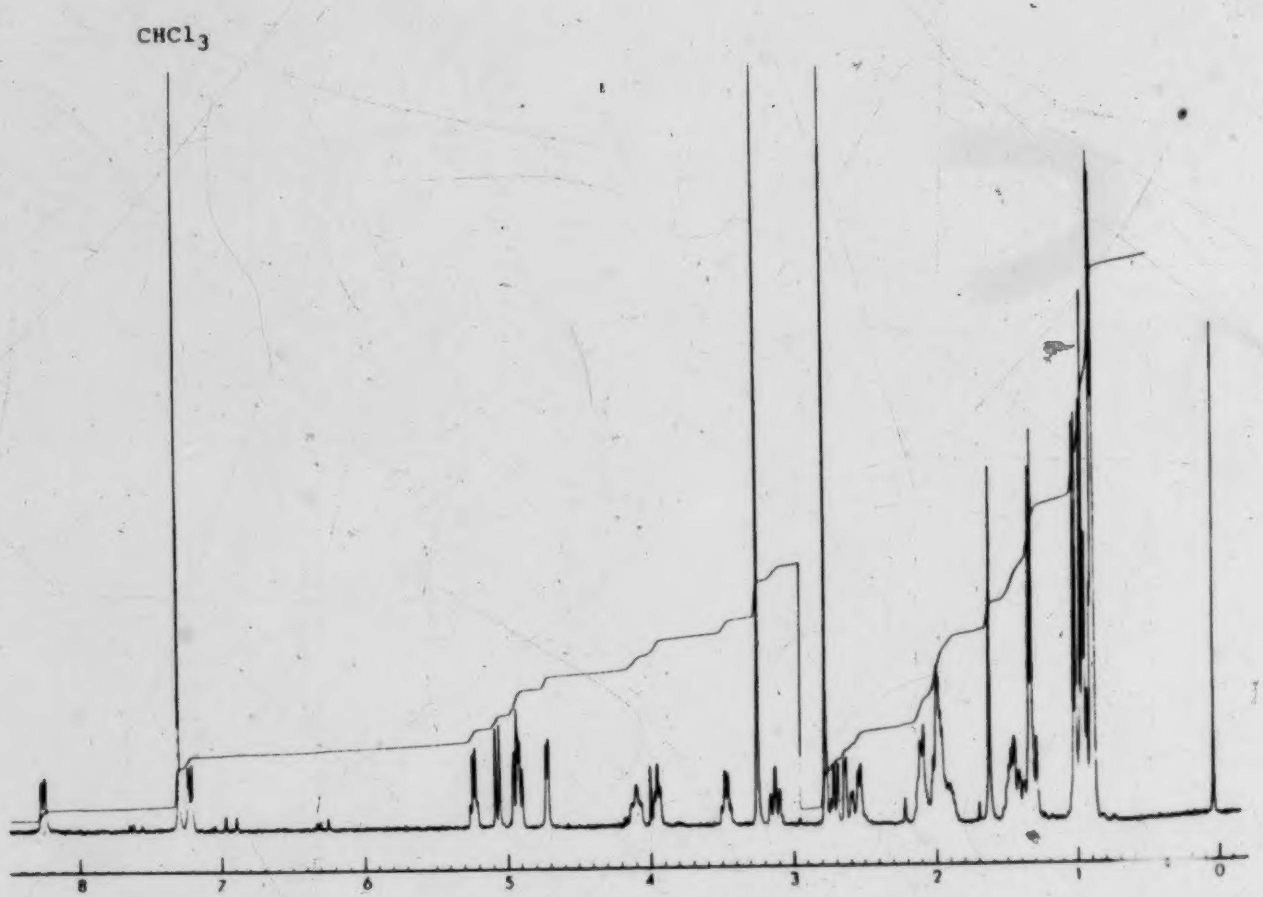
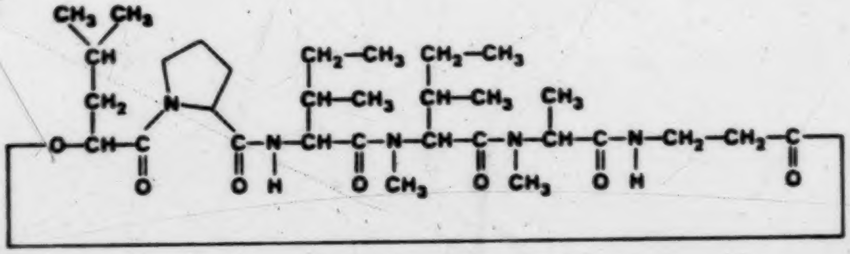


Figure 9. 400 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of homodestruxin B (**16**).

Table XII. Fragment ions in the HRMS of the methyl ester derivatives of destruxin B (**12**) and homodestruxin B (**17**).

12		**17**	
m/z	inf	m/z	inf
625	M^+	639	M^+
594	M^+-OCH_3	608	M^+-OCH_3
522	594-C_3H_6NO	537	608-C_3H_5NO
438	522-C_4H_6NO	452	537-C_4H_6NO
325	438-$C_6H_{11}NO$	325	452-$C_7H_{13}NO$
212	325-$C_6H_{11}NO$	212	325-$C_6H_{11}NO$
86	Base Peak	100	Base Peak

Homodestruxin B is a cyclodepsipeptide which has not been reported previously and its biological activity has not been investigated due to the small amount available to us.



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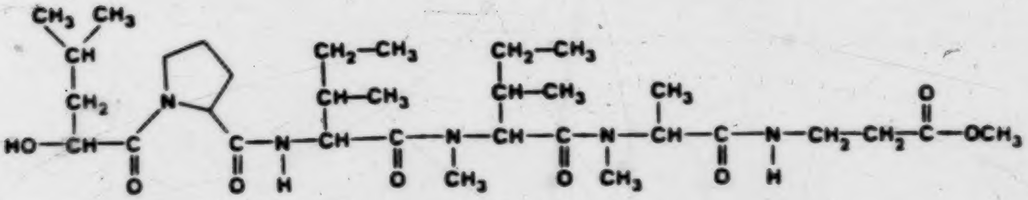
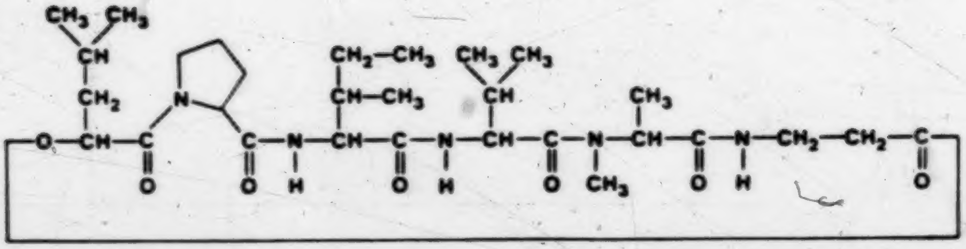


17

Desmethyldestruxin B (**18**), previously isolated from the fungus m. anisopliae,[33,34] was the third cyclodepsipeptide isolated from the organic extracts of A. brassicae culture filtrates. It is the most polar of the three (R_f 0.30, dichloromethane-acetone 70:30) and it was purified by preparative TLC from a mixture containing destruxin B (**13**), homodestruxin B (**16**), and desmethyldestruxin B (**18**).



18

Desmethyldestruxin B has the molecular formula $C_{29}H_{49}N_5O_7$ (mol. wt. 579), indicative of a destruxin B (**13**) structure but with one less methylene group. The main difference observed in the ^1H NMR of **18** (Figure 10) when compared to the ^1H NMR of the two other cyclodepsipeptides is the presence of an additional N-H as a broad doublet (J = 6 Hz) at δ6.24.

The signal at δ6.24 in the ^1H NMR of desmethyldestruxin B (**18**) was subjected to extensive decoupling experiments. In this manner the N-H proton was found to be coupled to a hydrogen at δ4.23 (dd, 6, 9.5 Hz) which in turn was coupled to a methine proton at δ2.05 (m). Irradiation of the multiplet at δ2.05 caused the collapse of two methyl doublets (δ1.03, 0.89; d, 7 Hz each) into singlets, thus confirming the methine proton as part of an isopropyl group. These decoupling experiments show that the N-H proton at δ6.24 is part of a valinyl (**xi**) group.

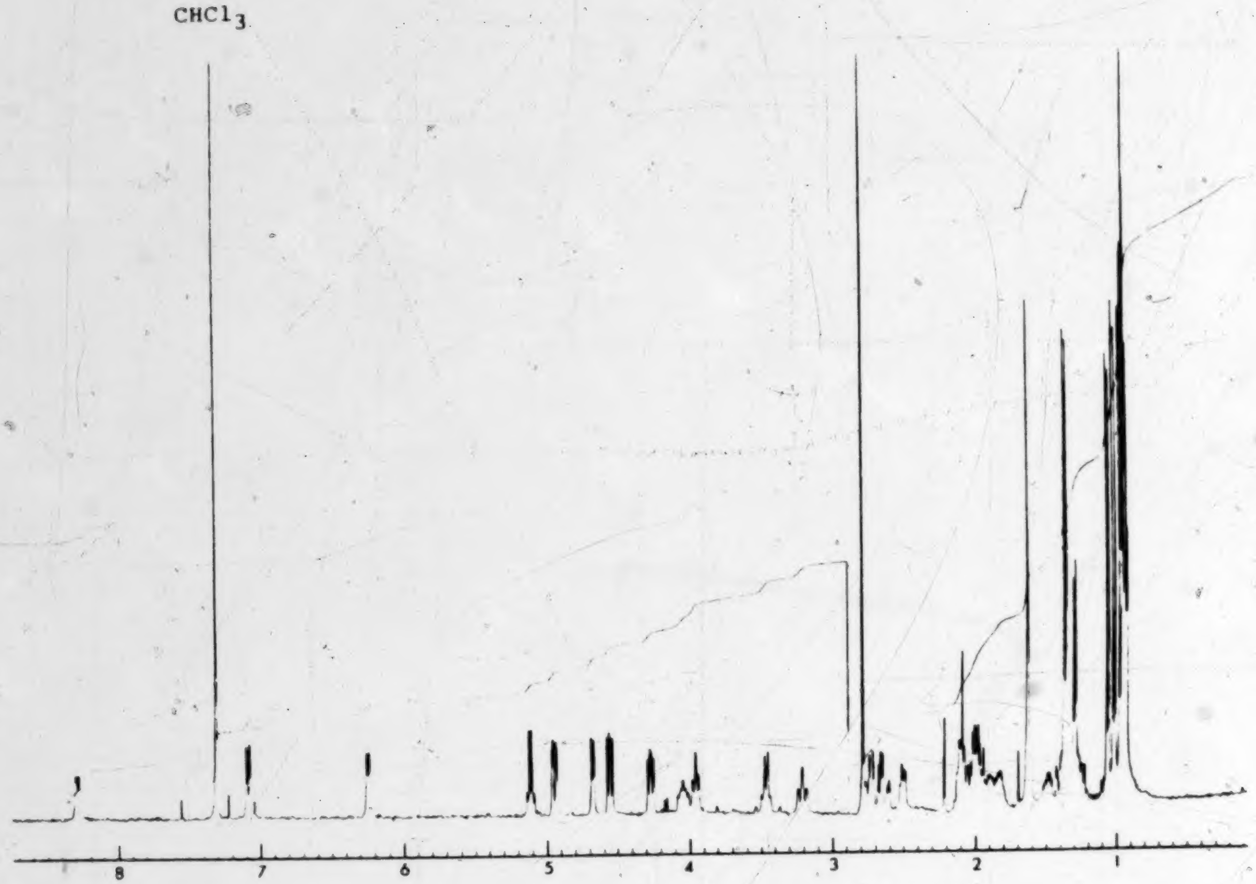
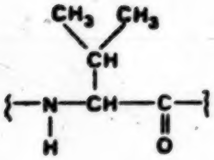
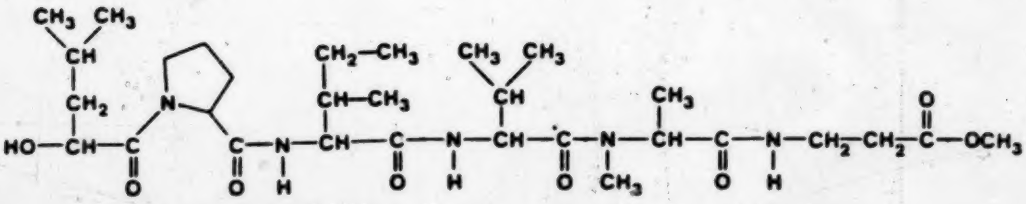


Figure 10. 400 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of desmethyldestruxin B (**18**).



21

Preparation of the hydroxymethyl ester derivative of desmethyldestruxin B (**19**) and comparison of its HRMS with that of destruxin B (**13**) (Table XIII) confirmed the presence of a valine moiety in the structure of the former. The loss of the N-methylvaline fragment in the HRMS of **12** is replaced by the loss of a fragment corresponding to a valinyl group (424 - C_5H_9NO) in the HRMS of **19**.



19

These data and comparison with that reported,[33,34] confirm structure **18** as the correct one for desmethyldestrxin B.

Component D, obtained as an amorphous white powder (mp. 100-104°) was separated from destruxin B (**13**) and purified by dry flash column chromatography (benzene-

Table XIII. Fragment ions in the HRMS of the methyl ester derivatives of destruxin B (**12**) and desmethyldestruxin B (**19**).

(**12**)		**19**	
m/z	inf	m/z	inf
625	M^+	611	M^+
594	M^+-OCH_3	580	M^+-OCH_3
522	594-C_3H_6NO	508	580-C_3H_6NO
438	522-C_4H_6NO	424	508-C_4H_6NO
325	438-$C_6H_{11}NO$	325	424-C_5H_9NO
212	325-$C_6H_{11}NO$	212	325-$C_6H_{11}NO$
86	Base Peak	70	Base Peak

acetone 80:20).

Compound **D** has a formula of $C_{15}H_{26}O_3$ (mol. wt. 254). The molecular weight of **D** was confirmed by CIMS (NH_3) where a peak at m/z 272 (M + 18) is observed. The IR spectrum of **D** shows hydroxyl (3299 cm^{-1}) and carbon-oxygen (1006 cm^{-1}) stretching bands, but lacks absorption in the carbonyl region suggesting that the three oxygens in the formula of **D** are present as alcohols and/or ethers. The fact that these three oxygens are present as hydroxyl groups was first indicated by fragments observed in the HRMS. An intense peak at M - 31 (m/z 223, $C_{14}H_{23}O_2$, 75%), resulting from the fragmentation of a primary alcohol,[36] is followed by peaks at m/z 205 ($C_{14}H_{21}O$, 27%) and m/z 187 ($C_{14}H_{19}$, 14%) corresponding to loss of one and two molecules of water respectively.

There are only two olefinic sp^2 carbon signals (δ 131.5, s; and δ 123.6, d) in the ^{13}C NMR of **D** indicating that of the three unsaturations implied by the formula of compound **D**, one is a double bond and two must be carbocyclic rings.

The ^1H NMR of **D** (Figure 11) displays three methyl singlets (δ 1.18, 1.08 and 0.88). This, along with the molecular formula, suggests that the compound may be a sesquiterpene.

Acetylation of compound **D** afforded a diacetyl

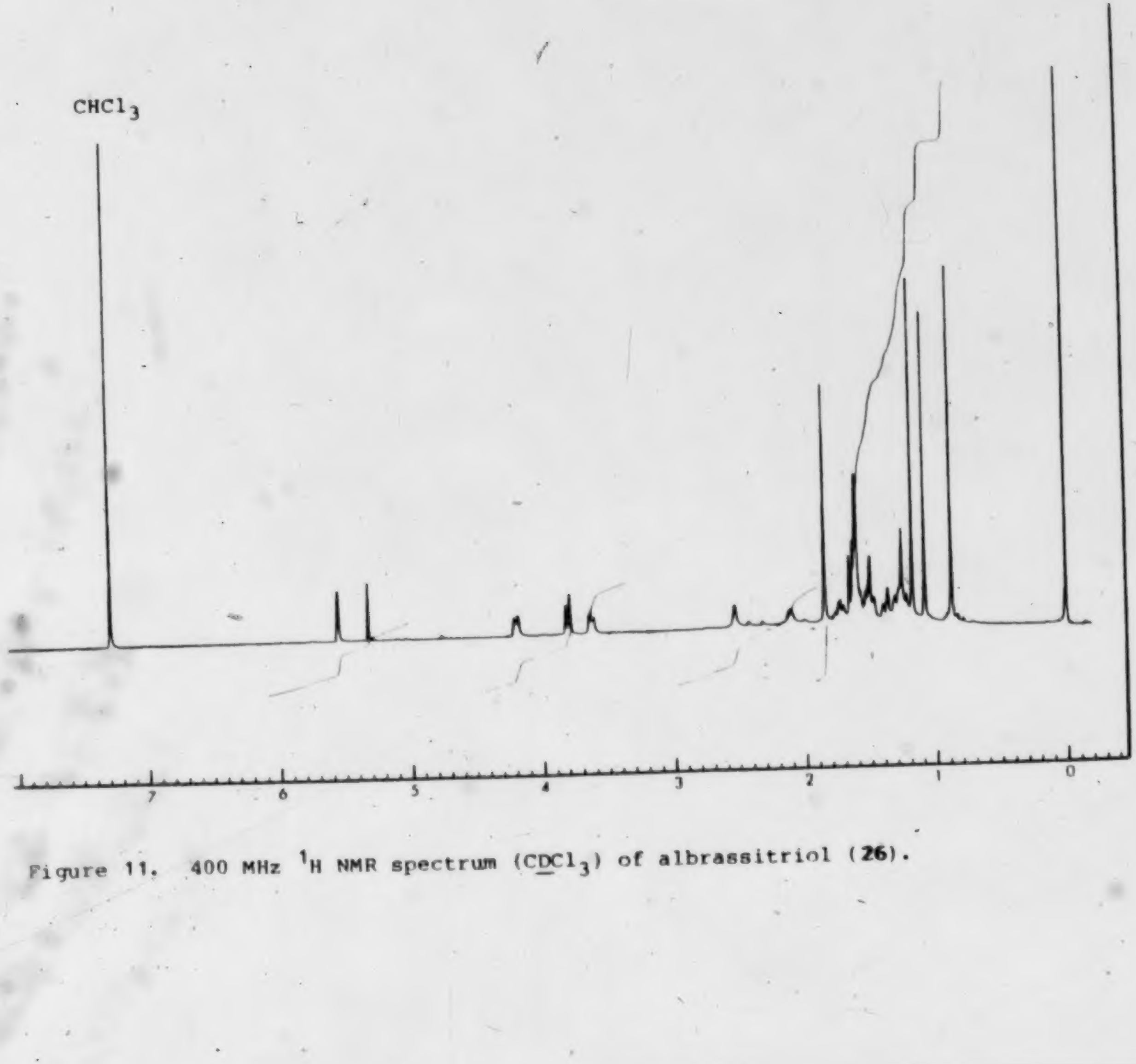
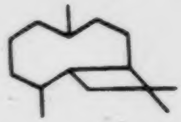


Figure 11. 400 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of albrassitriol (**26**).

derivative, **D'**, which displays two acetyl methyl signals in its ^{1}H NMR (δ 2.08 and 2.05, Figure 12). The IR spectrum of **D'** confirms the presence of the acetyl groups by the presence of an absorption band at 1733 cm^{-1}. In addition there is a strong hydroxyl absorption (3480 cm^{-1}), which suggests that the third oxygen of **D** is present as part of a tertiary hydroxyl.

In summary the data suggest that compound **D** is a bicyclic, trihydroxylated sesquiterpene. Four known sesquiterpenoid skeletons can be considered for **D** at this point: a caryophyllane (**20**), a chamigrane (**21**), a himachalene (**22**) or a drimane (**23**) skeleton.



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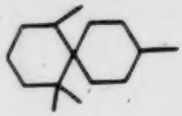

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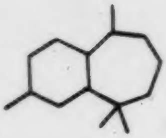

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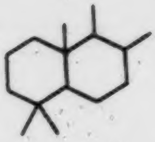


23

The nature of the acetylated alcohols was determined to be primary and secondary by comparison of the ^{1}H NMR spectra of **D** and **D'**. A one proton doublet at δ 3.78 (J =

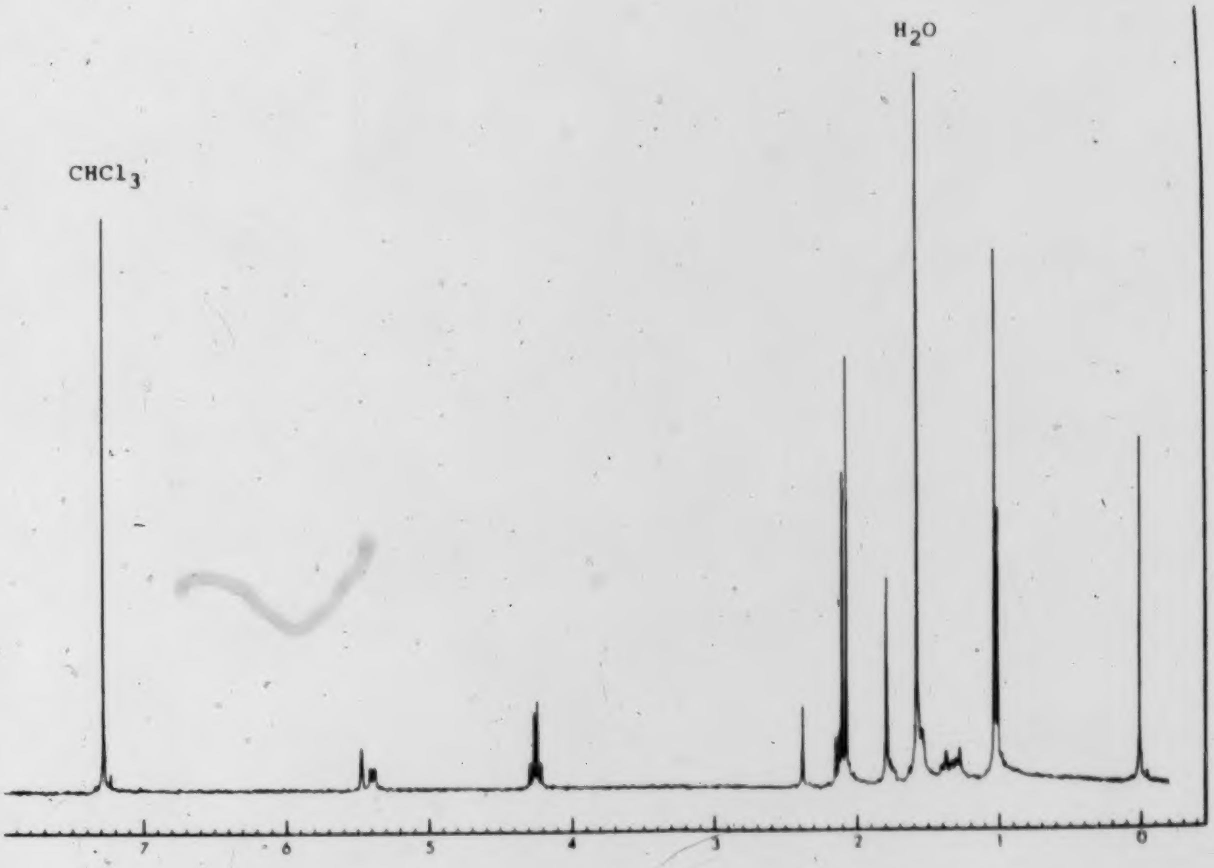
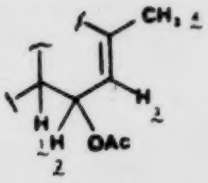


Figure 12. 400 MHz ^{1}H NMR spectrum ($\mathrm{C\underline{D}Cl_3}$) of O,O-diacetylalbrassitriol (**D'**).

11 Hz) in the ^1H NMR of **D** appears as a doublet of doublets of doublets (J = 1.5, 1.5, 10.5 Hz) at δ5.37 in the ^1H NMR of the diacetyl derivative **D'**. The magnitude of the "acylation shift"[56] (>1 ppm) is characteristic of that shown by carbinol protons of secondary alcohols upon acetylation.[56] The ^1H NMR of triol **D** displays methylene protons of a primary alcohol (δ4.17 and 3.60, both dd, J = 1, 10 Hz). These protons show a small downfield acetylation shift (δ4.26 and 4.21, both d, J = 10 Hz) in the ^1H NMR of **D'**. Primary alcohols usually show an "acylation shift" no larger than 0.5 ppm.[56] Thus **D** contains a primary, a secondary and a tertiary alcohol, and **D'** is its diacetyl derivative.

Decoupling experiments on the ^1H NMR spectrum of **D'** (Figure 12) show that the carbinol proton of the secondary acetoxyl group (δ5.37, ddd, 1.5, 1.5, 10 Hz) is coupled to a methine proton (δ2.12, d, 10 Hz), to an ethylenic hydrogen (δ5.44, dd, 1.5, 1.5 Hz), and to a vinylic methyl group (δ1.77, dd, 1.5, 1.5 Hz). These data are consistent with partial structure **xii** being present in **D'**.



xii

$J_{1,2}$ = 10 Hz

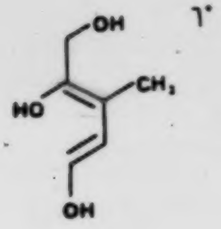
$J_{2,3}$ = 1.5 Hz

$J_{2,4}$ = 1.5 Hz

$J_{3,4}$ = 1.5 Hz

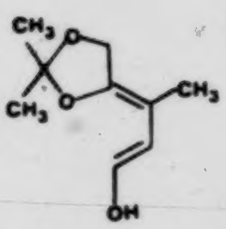
The presence of fragment **xii** is not possible in the chamigrane (**21**) skeleton, and thus this sesquiterpenoid skeleton need not be further considered.

In the HRMS of triol **D** a fragment ion occurs at m/z 154 ($C_{10}H_{18}O$, 10%). This ion cannot arise readily by fragmentation of a caryophyllane skeleton (**20**). Furthermore, the base peak in the HRMS of **D** occurs at m/z 130 ($C_6H_{10}O_3$). This ion, which contains all three oxygens of the molecule, can be accommodated by a drimane skeleton, in which case may have structure **xiii**.



xiii

The presence of a 1,2-diol in compound **D** was confirmed by the formation of an acetonide.[57] Triol **D** was treated with 2,2-dimethoxypropane and a catalytic amount of p-toluenesulfonic acid. The ^1H NMR of the product, **D***, showed two new methyl singlets at δ1.49 and 1.39, while in the HRMS of **D*** the expected fragment at m/z 170 (**xiv**) was observed.



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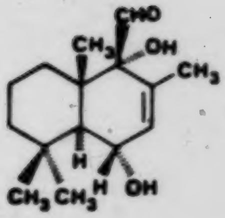

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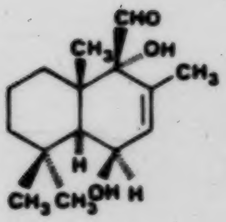

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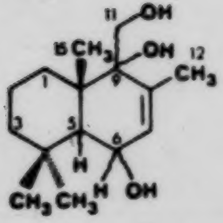


26

On the basis of the preceding spectroscopic data, and comparison with spectroscopic data reported for other naturally-occurring drimanes, e.g. mukaadial (**24**)[58] and cinnamodial (**25**),[59,60] compound **D** may be assigned structure **26**. The base peak in the mass spectrum is then accounted for by a retro-Diels Alder type fragmentation.

The ^{13}C NMR spectrum of **D** (Table XIV) gives further support to this structural assignment. Table XIV lists the chemical shifts of the 15 carbons, their multiplicities and their tentative assignment based upon reported values for similar natural products.[61,62]

The assignment of the trans configuration at the AB ring junction is based on the chemical shift observed for the angular methyl carbon in the ^{13}C NMR of compound **D** (16.8 ppm). It is known that the signal for the ring junction methyl in the trans isomer occurs 11-12 ppm further upfield than that of the cis isomer,[62,63] the latter usually resonating at approximately 30 ppm.[62,64] This also indicates that the proton at C-5 is axial (α-orientation). Since the coupling constant between H_5 and

Table XIV. ^{13}C NMR values of compound **26**.

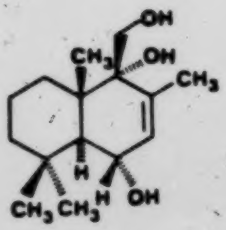
Carbon No.	ppm	Multiplicity
*1	42.9	t
2	18.6	t
*3	42.7	t
4	33.2	s
5	50.0	d
6	68.7	d
7	123.5	d
8	131.5	s
9	76.7	s
10	38.0	s
11	62.0	t
12	22.8	q
13	32.6	q
14	19.6	q
15	16.8	q

*May be reversed.

H_6 in the ^1H NMR of **26** is large (10 Hz), the two hydrogens must be in a trans diaxial arrangement. Thus H_6 is axial (β-orientation) and the C-6 hydroxyl group is equatorial (α-orientation). The J value observed for **26** is in agreement with the coupling constant reported for the same protons in the drimane mukaadial (**24**).[58] In cinnamodial (**25**), epimeric at C-6 with **26**, the H_5-H_6 coupling is 5 Hz.[59,60]

The stereochemistry of triol **26** at C-9 was determined by nuclear Overhauser enhancement (NOE) experiments. In similar compounds the α-hydroxyl configuration is reported to be biogenetically favored.[60] In the ^1H NMR of **26**, irradiation of the angular methyl singlet (C-15, δ1.08) resulted in a 4% enhancement of one of the C-11 methylene protons (δ4.17). This indicates that the $-CH_2$-OH group has a β-orientation and thus the C-9 hydroxyl must be α.

In addition, the α-orientation of the C-9 hydroxyl group was confirmed by observing the pyridine-induced solvent shifts when the ^1H NMR of the diacetyl derivative **D'** in $CHCl_3$-d_1 was compared with its ^1H NMR in C_5H_5N-d_5. The signal which showed the largest deshielding effect (Δ = -0.42 ppm, $\Delta = \delta_{CDCl_3} - \delta_{C_9D_5N}$) was the doublet (J = 10 Hz) corresponding to the α-proton at C-5. This is in agreement with the report that protons occupying positions 1,3-diaxial to a hydroxyl function experience deshielding effects of the order of 0.20 - 0.40 ppm in pyridine relative to chloroform.[65] Since H_5 is known to be α, the C-9 hydroxyl group must also be α. Thus the stereochemistry of **26** can be assigned as that depicted in the structure below.



26

To the best of our knowledge triol **26** is a new natural product and we have given it the name albrassitriol.

Two other drimane sesquiterpenes have been isolated and identified as metabolites of A. brassicae. The least polar of the two, component **G** (R_f 0.55, benzene-acetone 70:30) has a molecular formula $C_{15}H_{26}O_3$ (HRMS, mol. wt. 254), and is thus isomeric with albrassitriol (**26**).

The IR spectrum of **G** is very similar to that of compound **26**. It is dominated by strong hydroxyl absorption at 3400 cm^{-1}. The absence of absorption in the carbonyl region indicates that the three oxygens in **G** are present as alcohols.

The ^1H NMR of **G** (Figure 13) displays three methyl singlets (δ 1.08, 0.96, and 0.89), and the HRMS of **G** shows

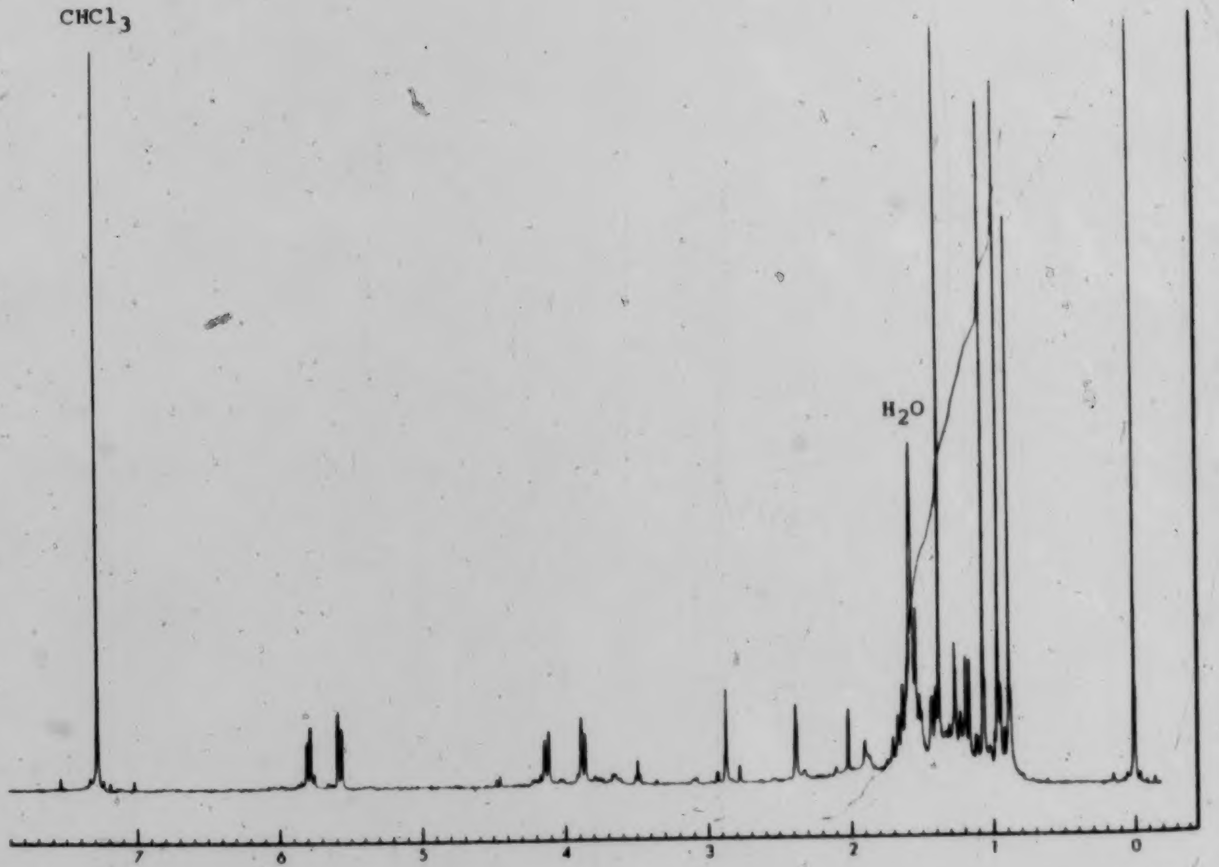
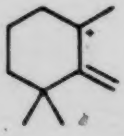


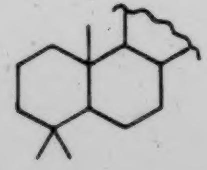
Figure 13. 400 MHz ^1H NMR spectrum ($C\underline{D}Cl_3$) of isoalbrassitriol (**28**).

an ion at m/z 137 ($C_{10}H_{17}$) consistent with a fragment having structure **xv**. This is characteristic of the



xv

fragmentation of terpenes having the bicyclic system **27**;[66,67] thus indicating that **G**, like albrassitriol (**26**), may be a trihydroxylated drimane.



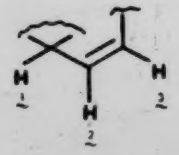
27

Acetylation of compound **G** afforded a monoacetyl derivative, **G'**, which showed a single acetyl methyl group singlet (δ 2.13) in its ^1H NMR.

In the IR spectrum of **G'**, absorption bands for both the ester carbonyl and hydroxyl groups are observed (1730 cm^{-1} and 3520 cm^{-1} respectively).

Comparison of the ^1H NMR of **G** with the ^1H NMR of its monoacetate derivative **G'**, indicates that the two doublets (J = 12 Hz each) at δ 4.11 and δ 3.86 in the ^1H NMR of **G**, assigned to hydrogens geminal to oxygen, experience a downfield shift of about 0.45 ppm (δ4.54 and δ4.33) in the ^1H NMR of **G'**. This "acylation shift"[56] value is characteristic of primary alcohols.[56] Thus compound **G** contains one primary and two tertiary hydroxyl groups.

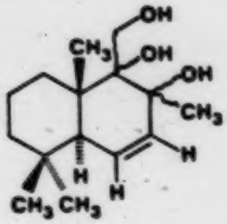
The ^1H NMR of compound **G** (Figure 13), unlike that of albrassitriol (**26**), displays two vinylic protons, one at δ5.77 (dd, 3, 10 Hz) and one at 5.55 (dd, 3, 10 Hz). Each proton was found by decoupling experiments to be coupled to a methine proton at δ2.37 (dd, 3, 3Hz). This suggests that partial structure **xvi** is present in **G**.



xvi

$J_{1,2} = 3$ Hz
$J_{1,3} = 3$ Hz
$J_{2,3} = 10$ Hz

A second difference between the ^1H NMR of **G** and the ^1H NMR of albrassitriol (**26**) is that **G** lacks a vinylic methyl group. On the other hand a three proton singlet at δ1.37, characteristic of a methyl geminal to oxygen, is observed.[56] On the basis of this spectral information structure **28** is tentatively proposed for compound **G**. Support for structure **28** is given by the HRMS of **G**. The

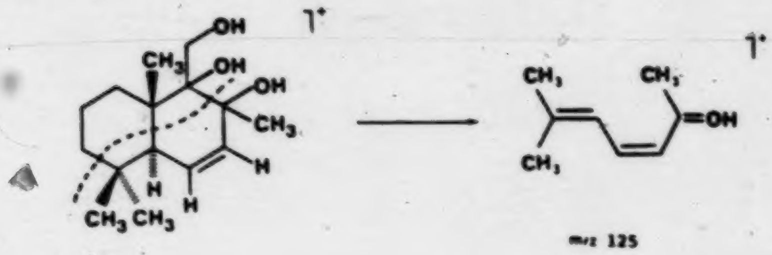

OH
CH₃
OH
OH
CH₃
H
H
H
CH₃ CH₃

28

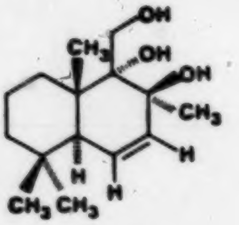
base peak at m/z 125 ($C_8H_{13}O$) can be accounted for as the fragment resulting from the cleavage of the 3-4, 8-9 and 10-5 (for numbering see **26**) carbon to carbon bonds as shown in Scheme VII.

Scheme VII

HRMS Fragmentation of G


OH
CH₃
OH
OH
CH₃
H
H
H
CH₃ CH₃
]⁺
CH₃
CH₃
CH₃
=OH
]⁺
m/z 125

Attempted formation of an acetonide of the monoacetyl derivative G' by treatment with 3,3-dimethoxypropane and p-toluenesulfonic acid,[57] resulted in the recovery of the starting material. This result strongly indicates that the vicinal tertiary hydroxyl groups are trans-diaxial. If we assume that the C-9 hydroxyl group of **28** has an axial orientation by analogy with albrassitriol, then the C-8 hydroxyl group should also be axial. Thus compound G, including stereochemistry, is assigned structure **28**.



28

An extensive search of the literature revealed that compound G is a new natural product. Since **28** is isomeric with **26**, compound G has been given the trivial name isoalbrassitriol.

The third drimane sesquiterpene, **H**, was isolated by preparative TLC (chloroform-methanol 95:5) of a biologically-inactive chromatography fraction.

Compound **H**, the most polar of the sequiterpenes (R_f 0.29, benzene-acetone 70:30) has a molecular formula of $C_{15}H_{24}O_3$ (mol. wt. 252) as determined by HRMS. The molecular weight was confirmed by the presence of intense peaks at m/z 270 (M+ 18) and 253 (M+ 1) in its CIMS (NH_3).

The ^1H NMR of **H** (Figure 14) shows three methyl singlets (δ 1.27, 1.16, and 0.92) and an olefinic proton

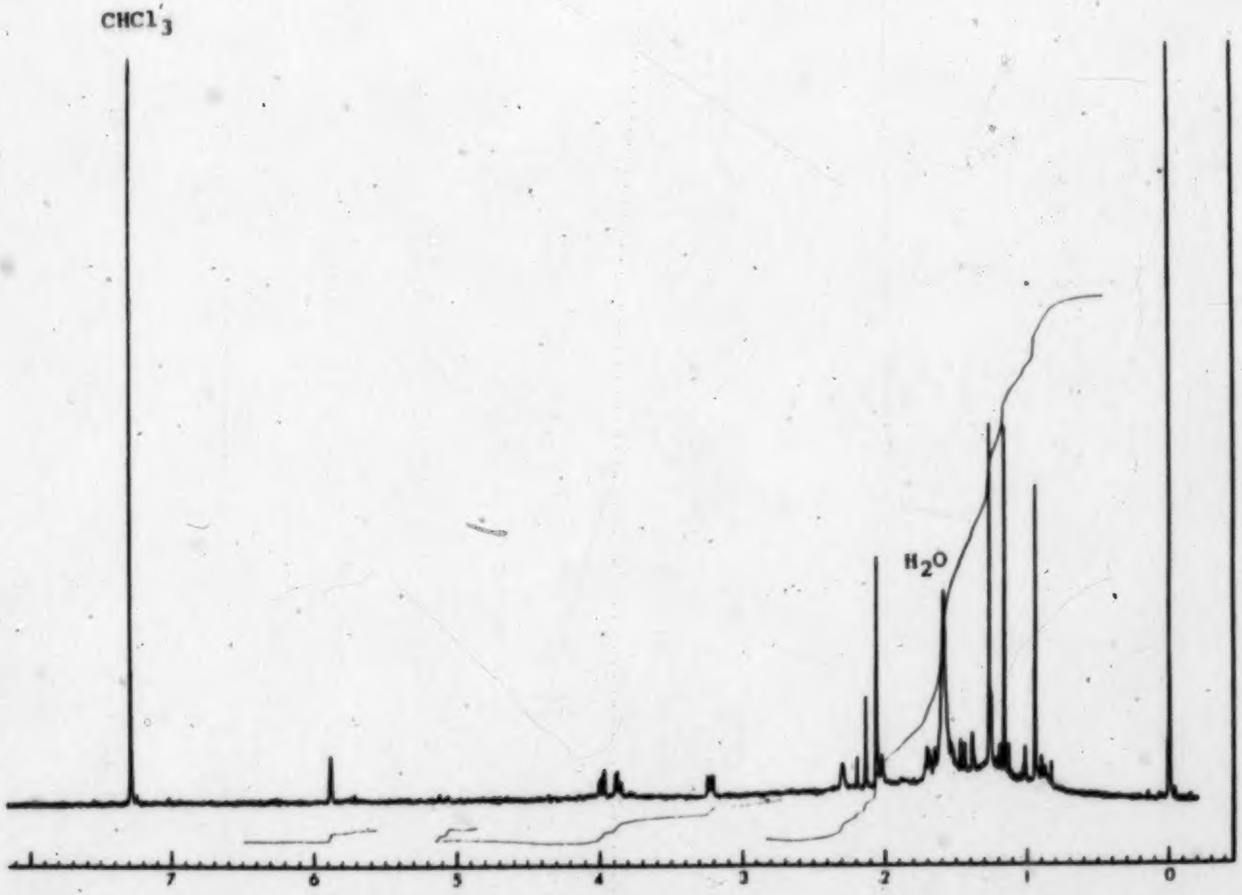
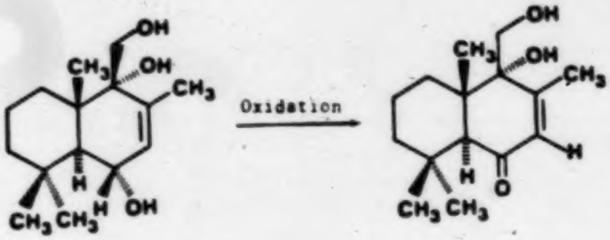


Figure 14. 400 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of deoxyuvidin B (**30**).

(δ5.86, bd, 0.5 Hz) as is observed in the ^{1}H NMR of the previously isolated drimane sesquiterpenes.

The IR spectrum of **H** shows characteristic absorption for an hydroxyl group (3400 cm^{-1}) as well as for the carbonyl group of an α,β-unsaturated system (1625 cm^{-1}). Evidence for the presence of an α,β-unsaturated ketone in **H** is given by the absorption at λ_{max} 235 nm (ε = 1763) in the ultraviolet (UV) spectrum.[36]

The number of unsaturations implied by the formula of **H**, together with the other spectral data suggest that **H** may have structure **29**. Compound **29** could arise by biological oxidation of albrassitriol (**26**).



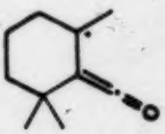
26 **29**

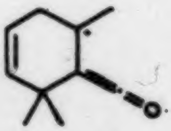
Equation 1

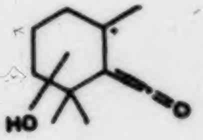
Treatment of compound **H** with a mixture of acetic anhydride/pyridine gave a diacetyl derivative, **H'**. The ^{1}H NMR of **H'** shows the acetyl methyls as a six proton singlet at δ2.07. Comparison of the ^{1}H NMR of enone **H** with the ^{1}H NMR of its diacetyl derivative **H'** reveals that three protons geminal to oxygen in **H** are shifted downfield in the ^{1}H NMR of **H'**. Two protons, both doublets of doublets, at δ3,97 (J = 3.5, 11 Hz) and δ3.86 (J = 6, 11 Hz), show downfield shifts of 0.36 ppm (δ4.33 and δ4.22). This suggests that **H** contains a primary alcohol. Another proton which appears as a doublet of doublets at 3.22 (J = 4.5, 10.5 Hz) in the ^{1}H NMR of **H** is shifted downfield 1.23 ppm to δ4.45 in the ^{1}H NMR of **H'**. This downfield "acylation shift"[56] is characteristic of that observed for a carbinol proton of a secondary alcohol.

These data indicate that **H** contains an enone and primary and secondary alcohols. Thus **H** does not have structure 29.

The presence of a secondary alcohol in **H** is most readily accommodated if it is located in ring A. Support for this hypothesis is provided by the fragmentation pattern observed in the HRMS of **H**. No peak at m/z 151 ($C_{10}H_{15}O$) corresponding to fragment ion **xvii**[62] was observed. Instead a peak at m/z 149 ($C_{10}H_{13}O$, fragment


xvii

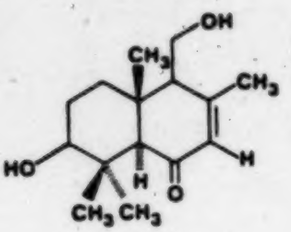

xviii



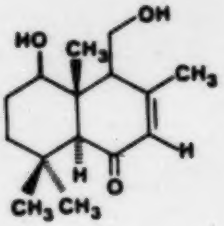
xix

xviii), which must result from the dehydration of fragment **xix**, is observed.

It is thus possible to propose structure **30** or **31** for compound **H**. Of the two possibilities, structure **30** is


OH
CH$_3$
CH$_3$
HO
H
O
H
CH$_3$ CH$_3$

30

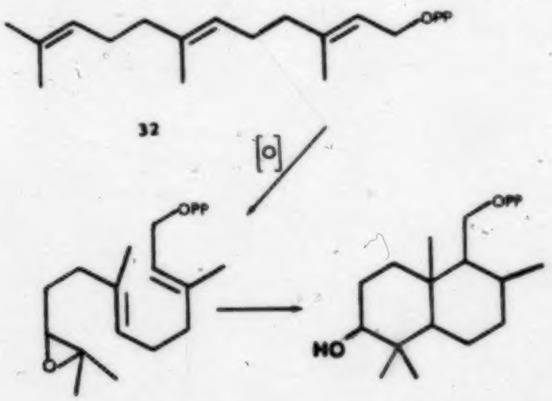

OH
HO
CH$_3$
CH$_3$
H
H
O
CH$_3$ CH$_3$

31

favored biogenetically since it is known that cyclization of epoxyfarnesylpyrophosphate (**32**) yields bicyclofarnesane sesquiterpenes hydroxylated at C-3[62,68,69,70] (**33**, Scheme VIII).

Scheme VIII

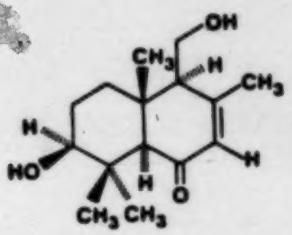
Cyclization of Epoxyfarnesylpyrophosphate (**32**)


OPP
[O]
OPP
OPP
HO

32

33

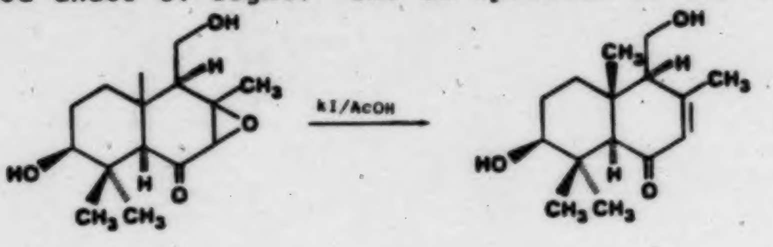
On this basis compound **H** is assigned structure **30**. The geminal proton of the secondary alcohol in the ^{1}H NMR of **30** appears as a doublet of doublets at δ3.22 (J = 4.5, 10 Hz). The coupling constants are consistant with an axial carbinol proton showing axial-equatorial and axial-axial coupling with the neighboring methylene.[62] Thus the C-3 hydroxyl group occupies the equatorial or β-orientation. In view of this the stereochemistry of **H** can be defined as shown below.



30

Compound **30** has spectral characteristics identical with those previously reported for deoxyuvidin B.[62] Deoxyuvidin B has not previously been obtained as a natural metabolite. It is the deoxygenation product of Uvidin B (**34**, Equation 2), a metabolite obtained from the mushroom Lactarium uvidus Fries.

Confirmation of the structure of **H** was obtained by transformation of **H** to Uvidin B (**34**). Natural deoxyuvidin B (**30**) was treated with basic hydrogen peroxide.[57] The reaction product showed two spots on TLC, the less polar component (R_f 0.63, benzene-acetone 70:30) was UV active while the more polar component (R_f 0.24, benzene-acetone 70:30) had a very similar R_f value to that of deoxyuvidin B (**34**) but could not be detected when the plate was observed under UV light. The IR spectrum of the reaction mixture showed carbonyl absorptions at 1667 cm^{-1} and 1720 cm^{-1}. This suggests a mixture containing an α,β-unsaturated ketone and an α,β-epoxyketone. Preparative TLC separation of the mixture afforded both components. The less polar and UV active component (**35**) displayed two carbonyl absorptions (1668 cm^{-1} and 1727 cm^{-1}) in its IR spectrum. Final identification was not possible because of the extremely small amounts involved.



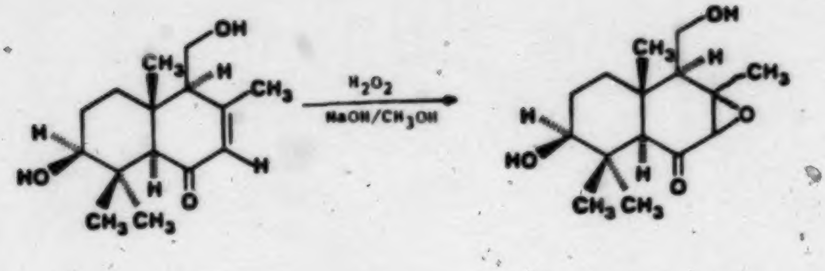
34 **35**

Equation 2

The more polar component showed a molecular ion at m/z 268 in its HRMS. This is the molecular ion expected for uvidin B (**34**). Strong fragment ions at m/z 149 and m/z 167 for fragments **xviii** and **xix** were also present. Final identification of the polar component as uvidin B

Scheme IX

Epoxidation of deoxyuvidin B (**30**)



OH
CH₃
H
CH₃
H
HO
H
O
H
CH₃ CH₃
H₂O₂
NaOH/CH₃OH
OH
CH₃
H
CH₃
O
H
HO
H
O
CH₃ CH₃

34

(**34**) was obtained by comparison with an authentic sample.* The IR spectrum of the polar component is superimposable on the IR spectrum of uvidin B (**34**). Thus the structure of compound **H** as that shown in **30** is firmly established.

Compound I was isolated as a milky oil after dry flash column chromatography purification of a biologically inactive chromatographic fraction. It has an R_f value between that of albrassitriol (**26**) and isoalbrassitriol (**28**) on TLC (R_f 0.43, benzene-acetone 70:30).

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*We thank Prof. P. Vita-Finzi (Instituto di Chimica Organica dell' Universita, Viale Taramelli 10, 271000 Pavia, Italy) for kindly providing us with a sample of uvidin B.

The molecular formula of compound **I** is $C_{15}H_{22}O_3$ (mol. wt. 250) by HRMS. The CIMS (NH_3) of **I** shows peaks at m/z 268 (M^+ + 18) and m/z 251 (M^+ +1) confirming the molecular weight as 250.

The IR spectrum of **I** shows hydroxyl (3335 cm^{-1}) and aromatic double bond stretching bands (1590, 1440 cm^{-1}). No carbonyl absorption is observed. The ^1H NMR of **I** (Figure 15) displays doublets at δ7.07 (J = 8 Hz) and 6.69 (J = 8 Hz) suggesting the presence of *ortho* aromatic protons.

Further evidence for the aromatic nature of **I** is given by its ^{13}C NMR (Figure 16) spectrum which shows that **I** contains six sp^2 carbons (δ159, 141, 125, 124, 122, 121). The chemical shift of the low field aromatic carbon (δ159) suggests that a phenol or aromatic ether may be present.[61,64]

These data account for four of the five unsaturation equivalents implied by the molecular formula of **I**, and suggest that the fifth unsaturation must be a carbocyclic ring since only six sp^2 carbons and no carbonyl carbons are observed in the ^{13}C NMR of **I**. Thus the carbon skeleton of compound **I** is defined as a benzene ring fused to a second ring and bearing two other substituents as

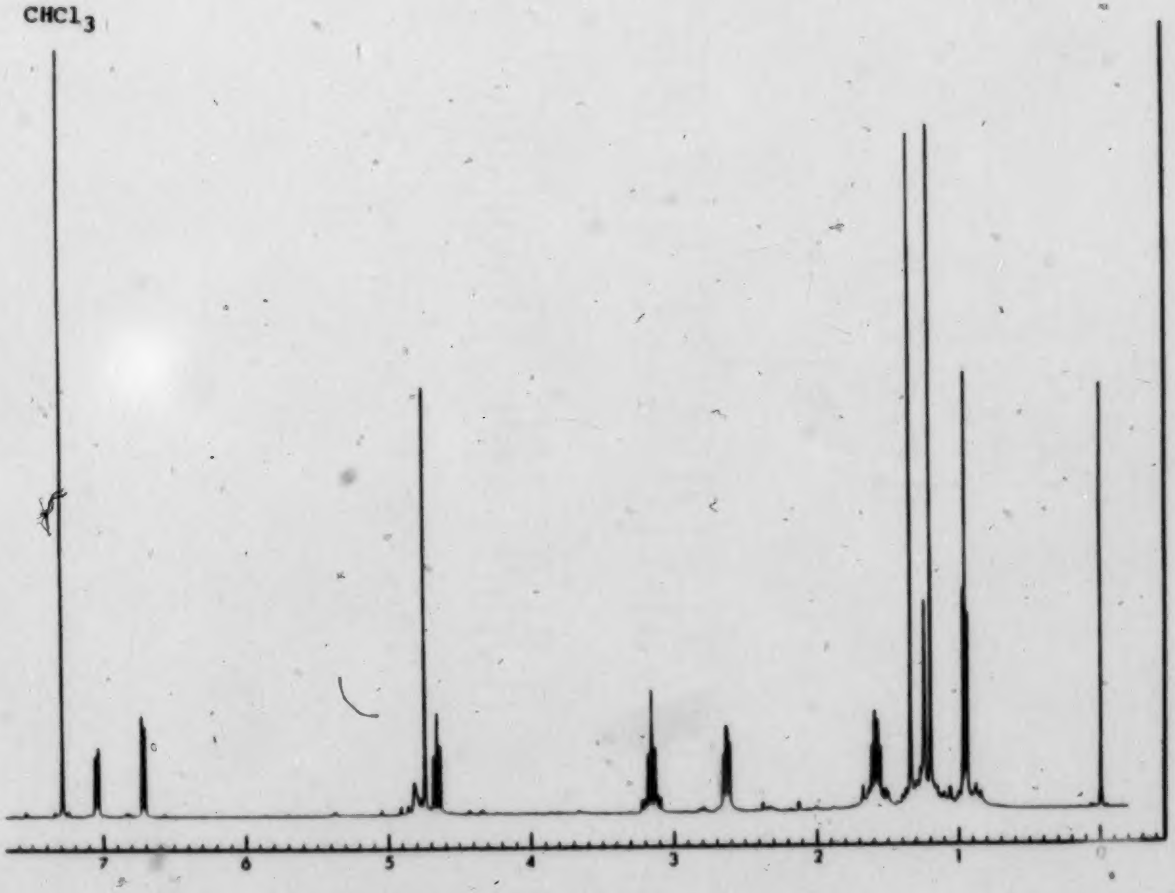


Figure 15. 400 MHz ^1H NMR spectrum ($C\underline{D}Cl_3$) of brassicadiol (38).

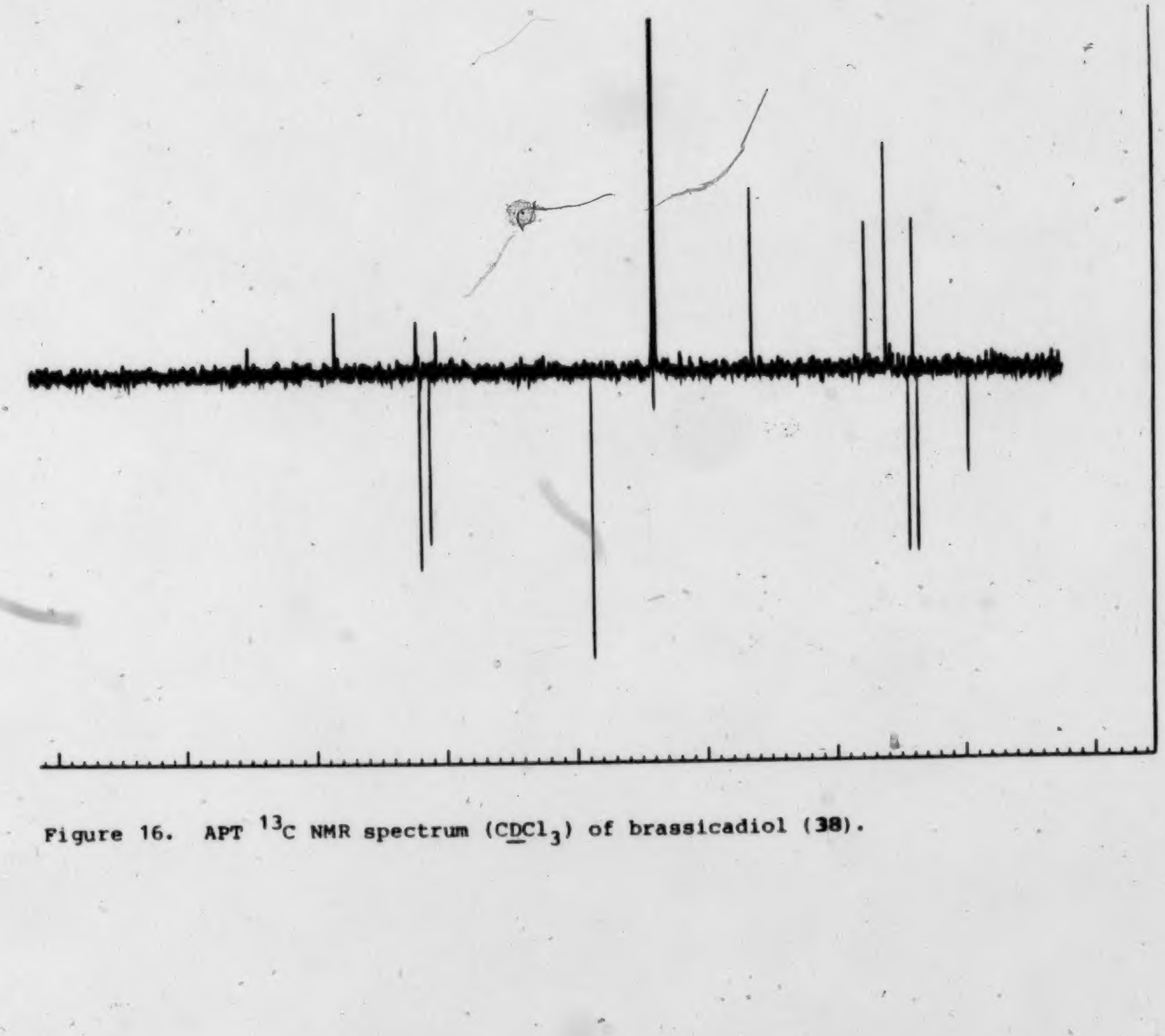
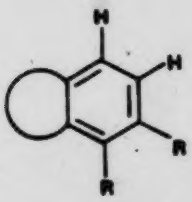
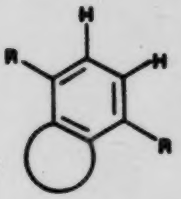


Figure 16. APT ^{13}C NMR spectrum ($C\underline{D}Cl_3$) of brassicadiol (**38**).

shown in partial structures **xx** and **xxi.**



xx



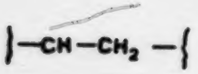
xxi

The ^1H NMR of **I** (Figure 15) shows two D_2O-exchangeable protons (δ 2.16 and 2.04). Its HRMS displays a base peak at m/z 174 ($C_{12}H_{14}O$) which corresponds to the loss of a $C_3H_8O_2$ fragment from the molecular ion. These data suggest that two of the oxygens present in **I** are hydroxyl groups while the remaining oxygen may be part of an ether moiety. This suggestion was corroborated by the fact that compound **I** forms a monoacetyl derivative, **I'**, upon treatment with acetic anhydride/pyridine. The IR spectrum of monoacetate **I'** shows strong absorptions characteristic of hydroxyl (3480 cm^{-1}) and acetyl carbonyl (1736 cm^{-1}) groups. Its ^1H NMR displays a single D_2O-exchangeable signal at δ 2.16.

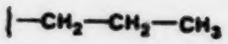
The acetoxyl group was shown to be on a primary carbon on the basis of the downfield shift observed in the ^1H NMR of **I'** with respect to the chemical shift of these same methylene protons in the ^1H NMR of **I** (δ 4.73 in **I** is shifted to δ 5.27 and 5.08 in **I'**).[56]

The HRMS of I' is similar to that of I in that it also shows a base peak at m/z 174. In this case the molecular ion loses a $C_5H_{10}O_3$ fragment. Confirmation that a tertiary hydroxyl group is present in I' was obtained when it was shown that only starting material was recovered when the monoacetate I' was subjected to treatment with Jones' reagent.[71]. With this information in hand it is possible to assign the three oxygens of I as a primary alcohol, a tertiary alcohol, and an ether linkage.

In addition to the tetrasubstituted aromatic ring (see partial structures **xx** and **xxi**), decoupling experiments performed on the ^{1}H NMR of I allowed the construction of two other partial structures. A doublet of doublets (J = 9, 9 Hz) at δ4.64 is coupled to methylene protons at δ3.17 and 3.11 (each dd, J = 9, 15 Hz), giving rise to partial structure **xxii**. An n-propyl group, partial structure **xxiii**, can be derived from the two proton signals at δ1.58 (ddd, J = 8, 8, 9 Hz) which is coupled to a methylene triplet (δ2.63, J = 8 Hz) and a methyl triplet (δ0.97, J = 8 Hz).

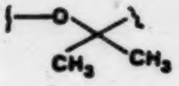


xxii



xxiii

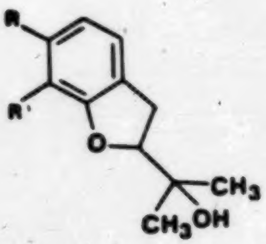
The methyl singlets at δ1.28 and 1.14 in the ^{1}H NMR of **I**, having chemical shifts characteristic of methyl groups geminal to oxygen,[36,56] suggest the presence of partial structure **xxiv**.



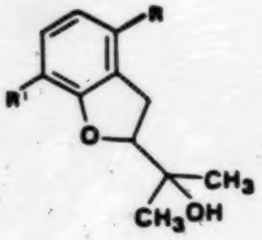
xxiv

Comparison of the chemical shifts of **I** with those reported for similar systems[56,64,71] suggested that both the n-propyl group (**xxiii**) and the primary alcohol are substituents on the aromatic ring.

The chemical shift of the methine proton of **xxii** is at rather low field in the ^{1}H NMR of **I** (δ4.64). There is no change in the chemical shift in the ^{1}H NMR of the monoacetyl derivative, **I'**, thus suggesting that this methine proton is geminal to an ether oxygen. The only oxygen available for ether formation is that located on the aromatic ring. Bonding of the methylene of fragment **xxii** to the aromatic ring, when considered with the preceding data, leads to the proposal of a dihydrobenzofuran type of structure (**36** or **37**) for compound **I**.



36



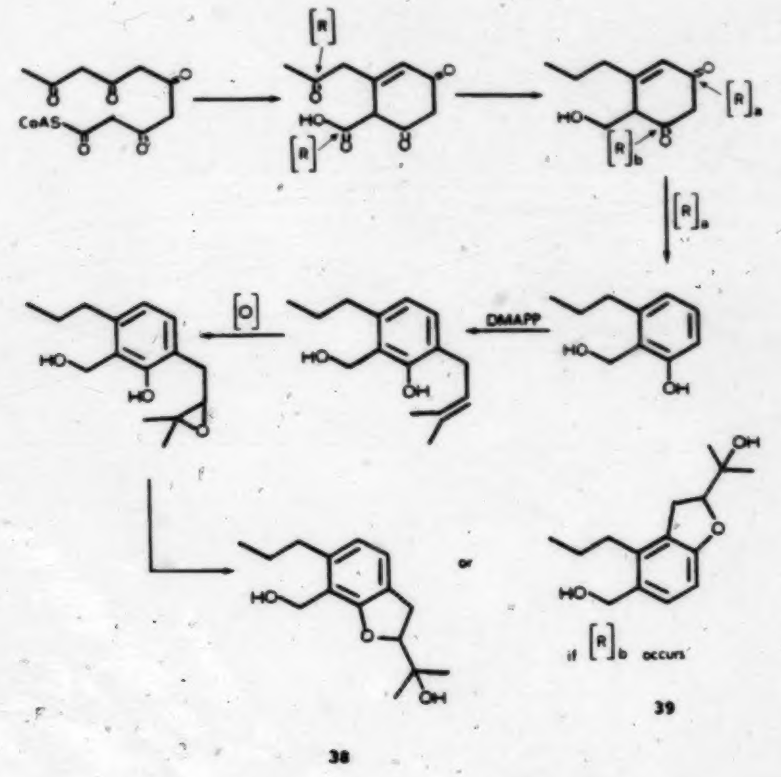
37

R = R' = $-CH_2OH$ or $n-C_3H_7$

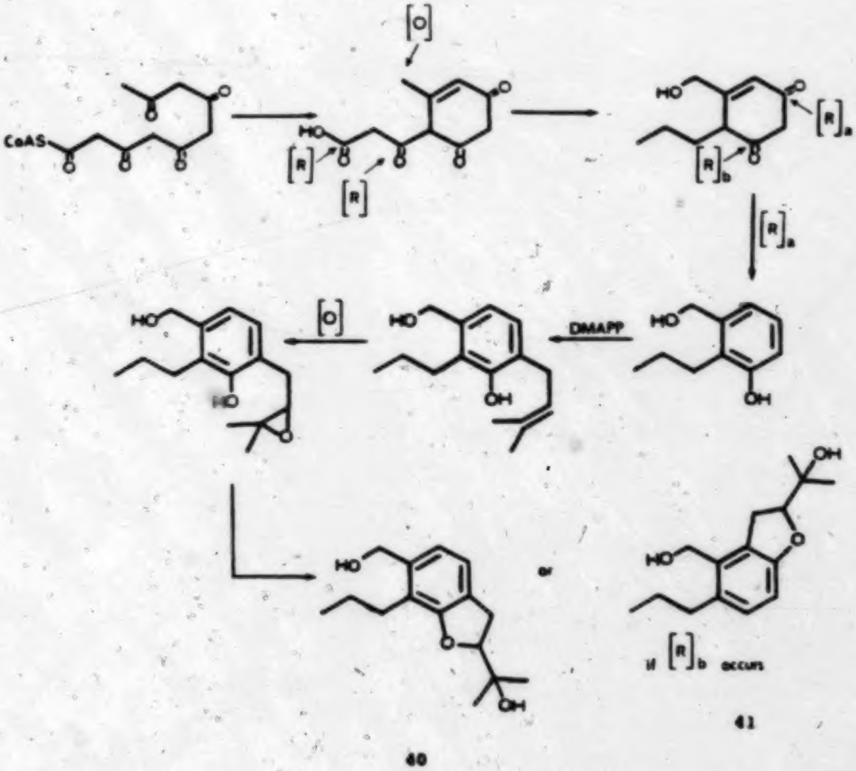
Scheme X illustrates that the probable biogenesis[73] of compound I favours an ortho orientation of the substituents in the aromatic ring (as in **36**). Structures of type **37** are biogenetically less favourable. At the same time biogenetic theory suggests that structures **38** or **39** and **40** or **41**, derived by a different "folding" of the polyketide chain (Schemes X and XI), are possible. Closer analysis of the ^1H NMR of monoacetate **I'** (scale: 2 Hz/cm) showed that the aromatic proton at $\delta 7.06$ is in fact a doublet of doublets of doublets ($J = 1, 1, 7.6$ Hz). Irradiation of the dihydrofuran methylene protons ($\delta 3.15$) resulted in the disappearance of the small couplings, thus indicating a long range coupling between the methylene and the aromatic protons. Of the four structures in Schemes X and XI (**38**, **39**, **40**, **41**) only **38** and **40** would give rise to such long range coupling. Accordingly structures **39** and **41** are eliminated.

Irradiation of the benzylic methylene group of the n-propyl group at δ 2.62 resulted in a sharpening of the

Scheme X. Possible biogenesis of compound I

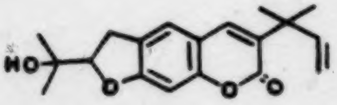

[R]
CoAS
HO
[R]
[R]a
[R]b
[R]a
DMAPP
[O]
HO
OH
or
if [R]b occurs
38
39

Scheme XI. Possible biogenesis of compound I

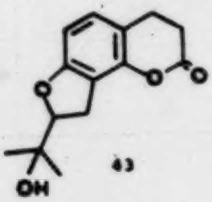

[O]
CoAS
HO
[R]
[R]
[R]a
[R]b
[R]a
DMAPP
[O]
HO
OH
or
if [R]b occurs
40
41

aromatic doublet at δ 6.69. This would be possible only in structure **38**, which is thus the one favoured for compound I.

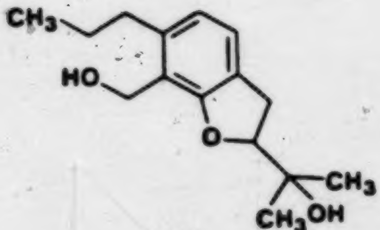
A structure of the type of **38** also explains the presence of the base peak at m/z 174 ($M^+ - C_3H_8O_2$) in the HRMS of compound I. This fragment may arise from the initial loss of acetone, a typical fragmentation observed in this type of system,[74] followed by loss of water (Scheme XII). Further evidence for the dihydrobenzofuran system **38** was obtained by comparing the chemical shift of the methine-methylene protons (partial structure **xxii**) in the ^1H NMR of compound I (δ 4.64 and 3.17, 3.11 respectively) with those reported for the same protons in heliettin[75] (**42**, δ 4.75 and 3.22), columbianetin[76,77] (**43**, δ 4.80 and 3.30), and marinesin[75] (**44**, δ 4.73 and 3.30). An extensive search of the literature has shown that compound I has not been previously reported. We have named this new natural product brassicadiol.



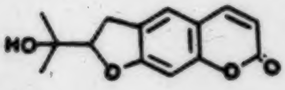
42



43



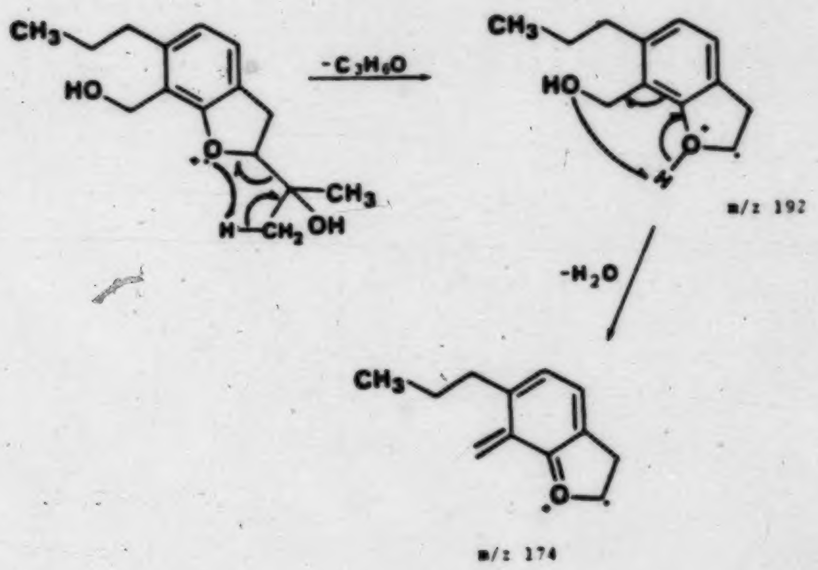
38



44

Scheme XII

MS fragmentation of compound I


CH3
HO
O
CH3
H
CH2
OH
-C3H6O
m/z 192
-H2O
m/z 174

EXPERIMENTAL

Water for fungal culturing was distilled in an all glass apparatus prior to use. Solvents for extraction and purification were distilled prior to use. Skellysolve B refers to Skelly Oil Company light petroleum, bp 62°-70°. E. Merck silica gel 60 (70-230 mesh) was used for column chromatography. Fractions were collected with an ISCO model 200 PUP fraction collector. For flash and "dry flash" column chromatography E. Merck silica gel 60 (230-400 mesh) was employed.

Analytical thin layer chromatography (TLC) was carried out on glass plates (75 × 25 mm or 75 × 50 mm) coated (approximately 0.3 mm) with silica gel G (Terochem, Edmonton) containing 1% electronic phosphor (General Electric, Cleveland) or E. Merck precoated TLC plates of silica gel 60 F-254 (0.25 mm thickness) or E. Merck DC-Alufolien, kieselgel 60 F-254 (0.2 mm thickness). The chromatograms were examined under ultraviolet light (254 or 350 nm) or by placing them in a chamber containing iodine vapour. Visualization was completed by spraying with 30% sulfuric acid or with a solution of 5% phosphomolybdic acid containing a trace of ceric sulfate in 5% sulfuric acid,* followed by careful charring with a heat gun.

Preparative thin layer chromatography was carried out on glass plates (20 × 20 cm, 10 × 20 cm, 5 × 7.5 cm) coated (0.35 mm thickness) with silica gel G (Terochem, Edmonton) containing 1% electronic phosphor (General Electric, Cleveland) or E. Merck precoated TLC plates of silica gel 60 F-254 (20 × 20 cm, 0.25 mm thickness). Materials were detected by visualization under an ultraviolet lamp (254 or 350 nm).

Melting points were determined on a Leitz Weztlar microscope heating stage equipped with Beevy (light intensity control) and Rewiz (temperature control) transformators, and are uncorrected.

Optical rotations were determined on a Perkin Elmer model 141 polarimeter.

Ultraviolet (UV) spectra were obtained on a Hewlett Packard model 8450A diode array spectrophotometer coupled to a Hewlett Packard 7470A plotter. Infrared (IR) spectra were recorded on a Nicolet 7199 FT interferometer.

High resolution electron impact mass spectra (HRMS) were recorded on an A.E.I. MS-50 mass spectrometer coupled to a DS-50 data system. Chemical ionization mass spectra

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*For composition see Appendix of Experimental.

(CIMS) and low resolution electron impact mass spectra (LRMS) were obtained using an A.E.I. MS-12 mass spectrometer. Fast atom bombardment (FAB) mass spectra were recorded using an MS-9 spectrometer coupled to a DS-55 data system. Data are reported as m/z (relative intensity). Unless diagnostically significant, peaks with intensities less than 20% of the base peak are omitted.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured on a Varian HA-100 spectrometer with a Digilab FTS/NMR-3 data system. High field ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker WH-200 or WH-400 spectrometers with an Aspect 2000 computer system. All nuclear magnetic resonance measurements employed tetramethylsilane (TMS) as an internal standard.

Centrifugation of the concentrated broth was carried out using a Dupont Instruments Sorvall RC-5B refrigerated superspeed centrifuge, equipped with a GSA rotor.

Amino acid analyses were performed in a Dionex D500 amino acid analyzer equipped with a Dionex 6A resin, using ninhydrin for the color developing system.

Culture of Alternaria brassicae (UAMH 4936*)

The strain of Alternaria brassicae used in these studies was isolated by Dr. J.P. Tewary, Department of Plant Science, The University of Alberta, from infected canola plants (Brassica campestris cv. Span) collected near Leduc, Alberta in July 1982. Stock cultures of the fungus were maintained in slant tubes on V-8 juice agar** at 4°C. Large scale production of A. brassicae was carried out as described below.

A. brassicae was asceptically transferred from slants to petri dishes containing the V-8 juice agar. The cultures were allowed to grow at room temperature for 10-14 days and then maintained at 4°C. Inoculum was prepared by blending a plate of A. brassicae with 200 mL of sterile, distilled water. The resulting suspension (10 mL) was used to inoculate liquid culture (1 L.).

In the early stages of the project, a growth study of A. brassicae was carried out. Growths of shake cultures on modified Czapek-Dox medium** and still cultures on V-8 juice medium with and without rose bengal** were compared. Of the three variations, still cultures grown on V-8 juice medium without rose bengal proved the best in terms of growth period, yield of metabolites, type of work up, and most importantly, production of bioactive metabolites.

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*Assession number given to Alternaria brassicae upon deposition at the University of Alberta mold herbarium.

**For composition see Appendix of Experimental.

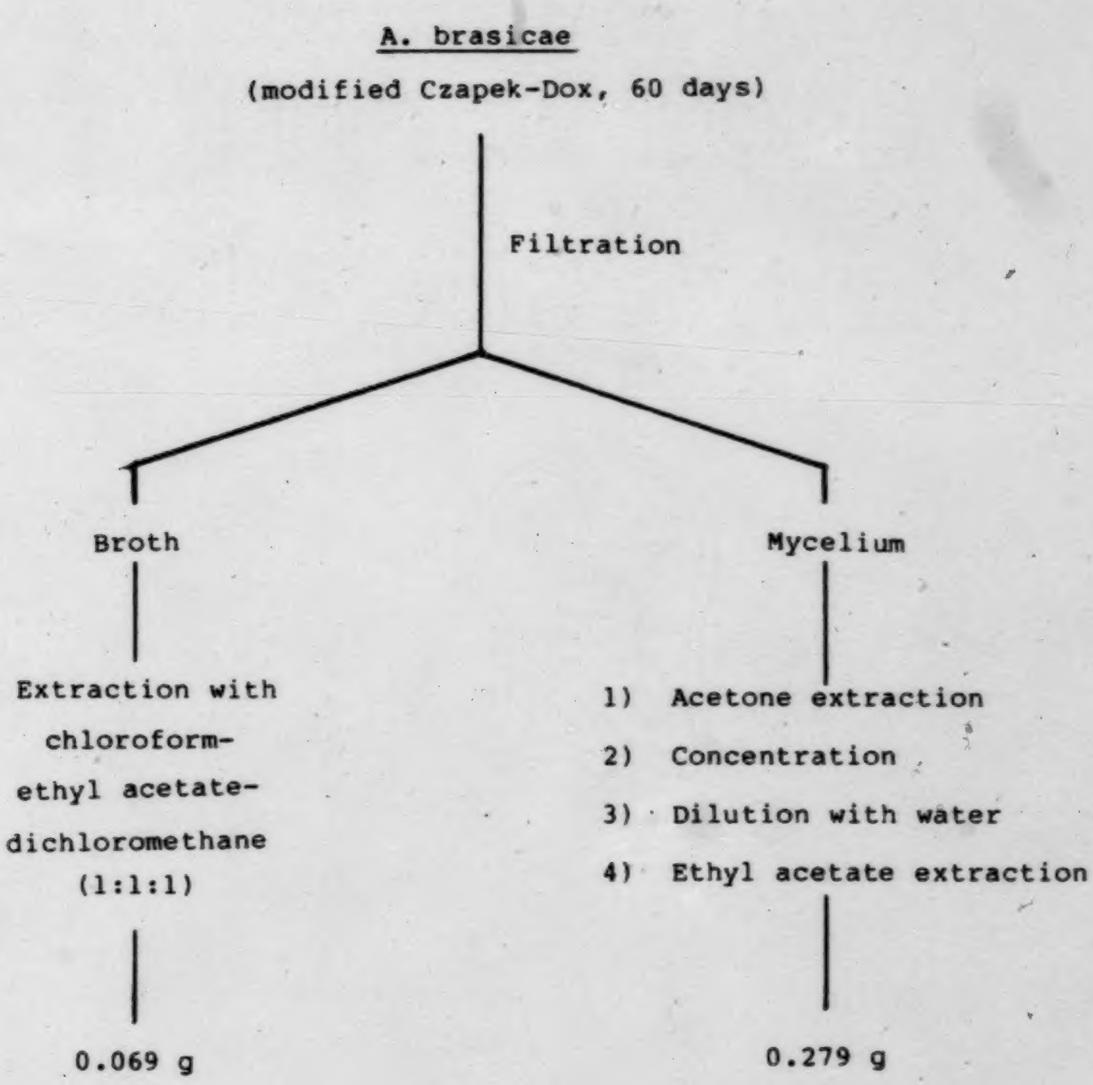
Work up and Extraction of Metabolites

(a) From Alternaria brassicae grown on modified Czapek-Dox medium

The fungus was grown for sixty days and worked up as shown in flow diagram 1. The mycelium was separated from the broth by gravity filtration through four layers of cheesecloth. The mycelial mat was left to dry in the fumehood for two days, then extracted with acetone for 24 h in a Soxhlet extractor. The acetone was removed at reduced pressure and the residue suspended in water. The resulting suspension was extracted by stirring with an equal volume of ethyl acetate (2×) for two hours.

The culture filtrate was concentrated under reduced pressure (temperature of the bath = 35°) to approximately 1/10 of its original volume. The concentrate was extracted with a mixture of chloroform:ethyl acetate:dichloromethane (1:1:1) for 24 h using a liquid-liquid extraction apparatus. The yields shown in flow diagram 1 are typical yields obtained from one liter of culture medium.

Flow Diagram 1


A. brasicae
(modified Czapek-Dox, 60 days)
Filtration
Broth
Mycelium
Extraction with chloroform-ethyl acetate-dichloromethane (1:1:1)
1) Acetone extraction
2) Concentration
3) Dilution with water
4) Ethyl acetate extraction
0.069 g
0.279 g

(b) From Alternaria brassicae grown on V-8 juice medium

The fungus was harvested after twenty days of growth. Flow diagram 2 describes the work up. The mycelium was separated from the broth, dried, and extracted as described previously.

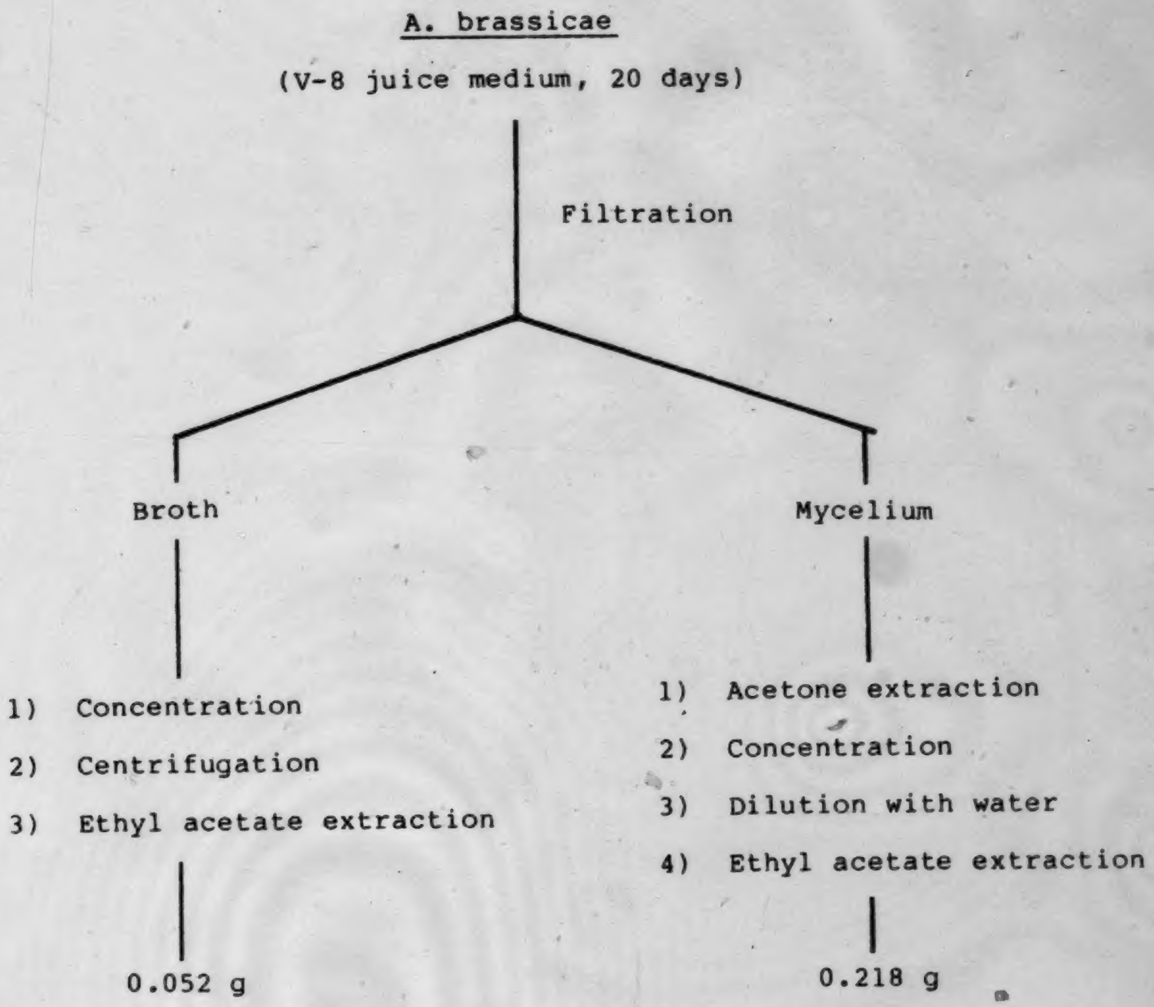
The broth was concentrated under reduced pressure (temperature of the bath = 35°) to approximately 1/20 of its original volume. A precipitate that appeared at this stage was removed by centrifugation (5000 rpm, 45 min, 20°C). The concentrated broth was then extracted with ethyl acetate for 24 h in a liquid-liquid continuous extractor. The yields shown in flow diagram 2 are average yields of crude extract obtained from one liter of culture broth.

Preliminary Fractionation of the Crude Extracts

(a) By column chromatography

The mycelial extract of A. brassicae grown in modified Czapek-Dox medium was fractionated by column chromatography. A 2 cm-diameter column was packed with silica gel (80 g) by suspension in chloroform. The crude extract dissolved in a minimum amount of chloroform-methanol 1% (ratio sample-adsorbent = ca. 1/250) was applied to the column. Separation was carried out using chloroform as eluant, and gradually increasing the

Flow Diagram 2


A. brassicae
(V-8 juice medium, 20 days)
Filtration
Broth
Mycelium
1) Concentration
2) Centrifugation
3) Ethyl acetate extraction
0.052 g
1) Acetone extraction
2) Concentration
3) Dilution with water
4) Ethyl acetate extraction
0.218 g

polarity of the solvent by the addition of methanol (i.e. 0.5%, 1%, 1.5%, 2.0%, etc., to 5%; 500 mL at a time). The flow rate was approximately twenty drops per minute. Fractions (5 mL) were collected using an automatic fraction collector. Fractions were combined on the basis of similarities of thin layer chromatograms to give 15-20 final fractions. Fractions K, L, S, and U were subjected to further separation.

(b) By partition/dry flash column chromatography

The crude extract was dissolved in a minimum amount of water-methanol (9:1), and the suspension partitioned successively between hexane, ethyl acetate, and n-butanol (3×, ratio solvent-suspension = 2:1). Each solvent extract was dried (anhydrous sodium sulfate), filtered and concentrated to afford the low, medium, and high polarity crude extracts respectively. Bioassay* of the three extracts showed that the medium polarity crude, which was the largest fraction, had the strongest biological activity. The organic crude extract was purified by dry flash column chromatography.* A typical separation can be described as follows: 350 mg of the crude extract were adsorbed on silica gel (ca. 2.5 g, ratio sample-silica gel = 1:7) and placed on top of a packed silica gel column (8 in long, 4 cm diameter). Expulsion of air and control of the flow rate (2 in/min) was done by passing Skellysolve B through the column. Separation was initiated by using Skellysolve B-acetone 90:10 as the initial eluant mixture. Gradient elution was done by increasing the amount of acetone (85:15, 80:20, 75:25, 70:30; 1 L of each). The manually collected fractions (30 mL) were monitored by TLC, and similar fractions were combined. Usually 15-20 final fractions were obtained, those showing biological activity were further purified.

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*For detailed description see Isolation and Identification of Metabolites.

Isolation and Identification of Metabolites

Leaf-spot bioassay

(i) Water-insoluble compounds. A 5% solution of the fraction to be tested is prepared in an organic solvent (chloroform-methanol 80:20). Approximately 20 μL of the test solution is adsorbed on silica gel (1 cm^2, 0.3 mm thickness) and allowed to dry. The impregnated silica gel is placed over a lightly scratched area of a canola leaf (Brassica napus L., var. westar) and wet with approximately 20 μL of distilled water. The leaf is maintained in a petri dish in a moist atmosphere at room temperature. Chlorosis is usually observed within 48 h.

(ii) Dry flash column chromatography. The method was developed in this laboratory to separate crude mixtures or complex fractions which are not soluble in the eluting solvent. The procedure is a combination of two techniques: dry column and flash chromatography. The chromatography column is packed as described by Still[47] omitting the sand at the top of the column. The sample adsorbed on silica gel (ratio sample-adsorbent 1:7) is loaded in a thin, even band, and then covered with approximately 2 1/2 in of sea sand. A non polar solvent is passed through until all of the air is expelled from the column, and the solvent flow rate is adjusted. Separation proceeds as described by Still[47] using the chosen solvent system as eluant. We have found that mixtures of Skellysolve B-acetone, ethyl ether-Skellysolve B, and benzene-acetone give excellent results for separation of *A. brassicae* and *M. nordinii* extracts. Compounds usually crystallize when the fractions are concentrated. The column length employed varied for complex separations (8 in) or for partial fractionations (4 in). This method was employed to effect separations of crude extracts (350 mg) or purification of reaction products (2 mg).

Isolation of the mixture of fatty acids

Fractions K (6 mg) and L (71 mg) from the purification of the mycelium extract presented a major component. This component was isolated (6 mg) by dry flash column chromatography (ethyl ether-Skellysolve B 1:1; column 4 in long, 0.5 cm diameter) purification of the combined fractions. TLC: R_f 0.43 (chloroform-methanol 95:5). IR ($CHCl_3$ cast): 2953, 2916, 2849, 1708 cm^{-1}. ^1H NMR (100 MHz, $C\underline{D}Cl_3$)*: δ0.9 (s), 1.28 (s), 2.02 (bd), 2.36 (t), 5.34 (t). HRMS: 382(4), 368(78), 354(34), 340(100), 326(7), 312(20), 284(3), 282(5), 256(1), 73(50), 60(37).

Esterification of the mixture of fatty acids

The mixture of fatty acids (5 mg) was dissolved in ethyl ether (2 mL) and allowed to stir at room temperature. Diazomethane was added to the solution until its yellow color persisted, and the TLC of the mixture showed no starting material. The product (3 mg) was subjected to gas chromatography-mass spectrometry (GC-MS) analysis (1 μL of sample, flow rate = 60 μL/min, T_1 = 50°/4 min, T_2 = 230°/32 min, rate = 8°/min, column = Apiezon L (10%)). Four major peaks were detected, and tentatively identified, on the basis of the molecular ion peaks observed, as C_{16}, C_{18}, C_{20}, and C_{22} methyl esters of the corresponding acids.

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*Integration was not possible.

Isolation of cerevisterol (**2**)

Fraction S (1.7 mg) obtained from the mycelium extract was recrystallized from chloroform. Cerevisterol (**2**, 0.8 mg) was obtained as a white, amorphous powder, mp 238°-242° (lit. = 256°). TLC = R_f 0.22 (Skellysolve B-ethyl acetate 80:20). IR ($CHCl_3$ cast): 340, 2951, 2923 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.61 (3H, s), 0.84 (3H, d, 7 Hz), 0.86 (3H, d, 7 Hz), 0.93 (3H, d, 7Hz), 1.02 (3H, s), 3.64 (1H, bs), 4.08 (1H, m), 5.19 (1H, dd, 8, 15 Hz), 5.21 (1H, dd, 8, 15 Hz), 5.38 (1H, bd, 5 Hz). HRMS: m/z calcd. for $C_{28}H_{44}O_2$ (P - H_2O, parent ion not seen) = 412.3341; found: 412.3372(88), 397(17), 394(56), 379(56), 376(17), 361(3), 340(4), 287(7), 269(30), 251(46), 245(3), 227(10), 215(10), 209(6), 177(2), 173(5), 159(8), 125(19). CIMS (NH_3): 413(37), 395(15), 377(100).

O-Acetylcerevisterol (**B'**)

Cerevisterol (0.8 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) were allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (10 mL) and worked up in the usual manner* to give the crude product (0.7 mg, yield = 79.5%). This product shows

one major component on TLC. Purification by dry flash column chromatography (Skellysolve B-ethyl acetate 90:10, 4 in long, 0.3 cm diameter) gave O-acetylcerevisterol (**B'**, 0.5 mg). TLC: R_f 0.50 (chloroform-methanol 95:5). IR ($CHCl_3$ cast): 3440, 3120, 2960, 2927, 1731, 1260, 1212, 800 cm^{-1}. CIMS (NH_3): 436(15), 394(13), 377(100).

Isolation of β-sitosteryl-β-D-glucoside (**6**)

Fraction U (4.2 mg) from the mycelium extract was purified by flash chromatography (chloroform-methanol 98:2, 4 in long, 0.5 cm diameter). Recrystallization from ethyl ether-methanol gave β-sitosteryl-β-D-glucoside (**6**) as a white powder (1.2 mg), mp = 271°-275°. TLC: R_f 0.09 (chloroform-methanol 95:5). IR ($CHCl_3$ cast): 3350, 2930, 2850, 1650 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3/C\underline{D}_3O\underline{D}$): δ0.68 (3H, s), 0.83 (3H, t, 6 Hz), 0.84 (3H, d, 6 Hz), 0.93 (3H, d, 6 Hz), 1.01 (3H, s), 3.31 (1H, m), 3.40 (1H, m), 3.50 (1H, bd, 5 Hz), 3.61 (1H, m), 3.66 (1H,bs), 3.77 (1H, dd, 2, 7 Hz), 3.87 (1H, dd, 2,7 Hz), 4.42 (1H, d, 4 Hz), 5.37 (1H, bs). HRMS: m/z calcd. for $C_{29}H_{50}O$ (P - sugar moiety, parent ion not seen): 414.3862; found:

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[*] Work up involves extraction with ethyl acetate (3×5 mL) followed by successive washings of the organic layer with water, HCl 5%, water, NaOH 5%, water, and NaCl satd. The organic layer is then dried (anh. sodium sulfate) and concentrated.

414.3891(7), 396(100), 303(4), 275(7), 273(2), 255(12), 231(2), 213(9), 163(1), 145(16), 119(10). CIMS (NH_3): 398(100), 397(63).

O,O,O,O-Tetraacetyl-β-sitosteryl-β-D-glucoside (**C'**)

β-Sitosteryl-β-D-glucoside (**6**, 1.2 mg) was treated with acetic anhydride (1 mL) and pyridine (0.5 mL), and allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (10 mL), extracted with ethyl acetate (3×10 mL), and worked up in the usual manner. The crude mixture showed a major component by TLC which was purified by dry flash column chromatography (Skellysolve B-ethyl acetate 95:15, 4 in long, 0.5 cm diameter), to give pure O,O,O,O-tetraacetyl-β-sitosteryl-β-D-glucoside (**C'**, 1.6 mg, yield = 76%). TLC: R_f 0.69 (chloroform-methanol 95:5). IR ($CHCl_3$ cast): 2961, 2928, 1751, 1741 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) = δ0.68 (3H, s), 0.83 (6H, d, 7.5 Hz), 0.86 (3H, t, 6.5 Hz), 0.93 (3H, d, 6.5 Hz), 0.99 (3H, s), 2.00 (3H, s), 2.02 (3H, s), 2.05 (3H, s), 2.08 (3H, s), 3.50 (1H, bs), 3.68 (1H, bs), 4.11 and 4.26 (1H e.a., AB part of an ABX system, 12, 5, 2 Hz), 4.60 (1H, d, 8 Hz), 4.96 (1H, dd, 8, 8 Hz), 5.08 (1H, dd, 8, 8 Hz), 5.22 (1H, dd, 8, 8 Hz), 5.37 (1H, bs). HRMS: m/z calcd. for $C_{29}H_{50}O$ (P – acetylated sugar moiety, parent ion not seen): 414.3862; found: 414.3864(0.5), 396(100), 382(13), 368(5), 255(7), 119(4). CIMS (NH_3): 762(17), 397(72), 396(100).

Isolation of destruxin B (**13**)

Separation of the medium polarity crude extract by dry flash column chromatography afforded 17 fractions. Strong biological activity was observed in fractions 10 (3.5 mg) and 11 (5.6 mg). Both showed by TLC the same major component. Fraction 11 was purified by recrystallization from Skellysolve B-acetone to give destruxin B (**13**, 2.8 mg) as white needles, mp 225°-227° (lit. 238°). TLC: R_f 0.23 (dichloromethane-acetone 90:10), 0.73 (dichloromethane-acetone 70:30), 0.49 (chloroform-methanol 95:5). IR ($CHCl_3$ cast): 3360, 3290, 1730, 1670, 1630, 1515 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ 8.19 (1H, d, 9 Hz), 7.17 (1H, d, 9 Hz), 5.19 (1H, q, 6.5 Hz), 4.94 (1H, d, 11 Hz), 4.84 (1H, dd, 3, 10 Hz), 4.67 (1H, bd, 7 Hz), 4.05 (1H, dddd, 13.5, 10, 5, 2 Hz), 3.91 (1H, bt, 9 Hz), 3.43 (1H, bq, 9 Hz), 3.20 (3H, s), 3.08 (1H, dddd, 13.5, 11.5, 2, 1.5 Hz), 2.73 (3H, s), 2.66 (1H, ddd, 18, 11.5, 2 Hz), 2.59 (1H, ddd, 18, 5, 2 Hz), 2.49 (1H, m), 2.32 (1H, dsept., 11, 6.5 Hz), 2.08 (1H, bs), 1.90 (7H, m), 1.30 (3H, d, 7 Hz), 0.99 (3H, d, 6.5 Hz), 0.94 (3H, d, 6 Hz), 0.93 (3H, d, 6 Hz), 0.89 (3H, d, 6.5 Hz), 0.86 (3H, d, 7 Hz), 0.86 (3H, t, 7.5 Hz). ^{13}C NMR ($C\underline{D}Cl_3$): 173.70 (s), 173.55 (s), 171.08 (s), 170.93 (s), 169.66 (s, 2×), 71.95 (d), 60.71 (d), 58.13 (d), 55.46

(d), 53.66 (d), 46.44 (t), 38.95 (t), 37.53 (d), 34.42 (t), 33.21 (t), 30.80 (d), 28.85 (t), 28.03 (d), 27.23 (q), 24.49 (t), 24.41 (q), 24.10 (t), 24.32 (q), 21.49 (q), 20.00 (q), 19.62 (q), 15.37 (q), 15.21 (q), 11.32 (q). HRMS: m/z calcd. for $C_{30}H_{51}N_5O_7$ (M^+): 593.3789; found: 593.3798(7), 565(4), 536(30), 508(23), 451(22), 423(22), 86(100), 70(60). FABMS: 1188(0.1), 594(22)

Destruxin B-methylester (**12**)

Destruxin B (**13**, 7 mg), methanol (2 mL), and a catalytic amount of potassium carbonate were allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (20 mL) and extracted with dichloromethane (4×6 mL). The organic layer was separated, dried (anh. sodium sulfate), and concentrated to yield 6 mg of crude product containing a single compound, more polar than the starting material, on TLC. Dry flash column chromatography (benzene-acetone 70:30, 4 in long, 0.4 cm diameter) purification of the crude afforded 4 mg (yield = 54%) of pure destruxin B-methylester (**12**). TLC: R_f 0.36 (dichloromethane-acetone 70:30), 0.15 (benzene-acetone 70:30). IR ($CHCl_3$cast): 3420, 3312, 2950, 1739, 1680, 1631, 1537 cm^{-1}. ^1H NMR

(400 MHz, $C\underline{D}Cl_3$)*: δ 7.68 (bt, 5 Hz), 7.41 (bd, 9 Hz), 6.99 (bd, 9 Hz), 6.47 (bt, 5 Hz), 5.20 (d, 11 Hz), 3.62 (s), 3.61 (s), 3.19 (s), 3.00 (s), 2.95 (s), 2.71 (s), 1.29 (d, 8 Hz), 1.21 (d, 8 Hz). HRMS: m/z calcd. for $C_{31}H_{55}N_5O_8$ (M^+): 625.4050; found: 625.4034(0.7), 594(0.7), 522(1), 438(25), 325(7), 212(22), 86(100), 70(42).

O-Acetyldestruxin B-methylester (**E"**)

The methanolysis product of destruxin B (**12**, 4 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) were allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (20 mL) and extracted with ethyl acetate (3× 10 mL). Work up of the organic extract in the usual way gave the crude product (5 mg). Purification by passing the crude through silica gel gave 2.9 mg (yield = 68%) of pure acetylated destruxin B-methylester (**E"**). TLC: R_f 0.64 (dichloromethane-acetone-methanol 70:26:4). IR ($CHCl_3$ cast): 3314, 2961, 1739, 1679, 1641, 1531, 1441, 1241 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$)*: δ7.65 (bt, 6 Hz), 7.46 (bd, 9 Hz), 7.35 (bd, 9 Hz), 6.55 (bt, 5 Hz), 5.29 (d, 11 Hz), 5.18 (d, 11 Hz), 3.67 (s), 3.28 (s), 3.10 (s), 2.97 (s), 2.78 (s), 2.14

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*Not all signals are listed since the compound is a mixture of two diastereomers.

(s), 2.12 (s), 1.35 (d, 8 Hz), 1.27 (d, 8 Hz). LRMS: 667(2), 636(2), 564(2), 480(52), 367(44), 254(51), 86(100), 70(73).

Isolation of homodestruxin B (**16**, cyclo-D-α-hydroxy-γ-methylvaleryl-L-prolyl-L-isoleucyl-N-methyl-L-isoleucyl-N-methyl-L-alanyl-β-alanyl)

Fractions 11 (5.6 mg) and 14 (3.7 mg), obtained by purification of the medium polarity extract, showed two major components on TLC. Preparative TLC (20×20 cm, chloroform-methanol 98:2, 3×) purification of the fractions yielded each component in pure form. The more polar compound was identified as destruxin B (**13**). The less polar compound was obtained as an amorphous white powder (2.5 mg), mp 202°-204°, and identified as homodestruxin B (**16**). TLC: R_f 0.27 (dichloromethane-acetone 90:10), 0.85 (dichloromethane-acetone 70:30), 0.56 (chloroform-methanol 95:5). IR ($CHCl_3$ cast): 3390, 3300, 2962, 1730, 1672, 1632, 1539, 1515, 1442 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ8.21 (1H, d, 9.5 Hz), 7.16 (1H, d, 9 Hz), 5.15 (1H, q, 6.5 Hz), 4.99 (1H, d, 11 Hz), 4.87 (1H, dd, 10.5, 3 Hz), 4.33 (1H, dd, 7, 9.5 Hz), 4.64 (1H, bd, 7.5 Hz), 4.02 (1H, dddd, 13.5, 10, 5, 2 Hz), 3.87 (1H, bt, 8.5 Hz), 3.39 (1H, bq, 9 Hz), 3.16 (3H, s), 3.03 (1H, dddd, 13.5, 11.5, 2, 1.5 Hz), 2.69 (3H, s), 2.62 (1H, ddd,18, 11.5, 2 Hz), 2.53 (1H, ddd, 18, 5, 1.5 Hz), 2.45

(1H, m), 1.25 (3H, d, 7 Hz), 0.93 (3H, d, 7 Hz), 0.89 (3H, d, 7 Hz), 0.88 (3H, t, 7 Hz), 0.81 (3H, t, 7 Hz), 0.80 (3H, d, 7 Hz). HRMS: m/z calcd. for $C_{31}H_{53}N_5O_7$ (M^+): 607.3945; found: 607.3960(4), 550(21), 508(19), 451(20), 423(19), 100(100), 86(11), 70(48). CIMS (NH_3): 609(34), 608(100).

Homodestruxin B-methylester (**17**, α-hydroxy-γ-methylvaleryl-L-prolyl-L-isoleucyl-N-methyl-isoleucyl-N-methyl-L-alanyl-β-alanyl-methylester)

Homodestruxin B (**16**, 1 mg), methanol (1 mL), and a catalytic amount of potassium carbonate were allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (10 mL) and extracted with dichloromethane (3×5 mL). The organic layer was separated, dried (anh. sodium sulfate), and concentrated to produce crude methanolysis product (**17**, 0.9 mg, yield = 85%). TLC: R_f 0.52 (dichloromethane-acetone-methanol 70:26:4). IR ($CHCl_3$ cast): 3310, 2959, 1738, 1679, 1630, 1535, 1482 cm^{-1}. HRMS: m/z calcd. for $C_{31}H_{53}N_5O_7$ (P − CH_3OH, parent ion is not seen): 607.3945; found: 607.3945(0.2), 537(0.3), 452(12), 325(3), 212(16), 100(100), 86(10), 70(42).

Isolation of desmethyldestruxin B (**18**)

Fraction 13 (27.7 mg) from the purification of the medium polarity crude extract contained a mixture of homodestruxin B (**16**), destruxin B (**13**), and a third cyclodepsipeptide. Preparative TLC (20×20 cm, chloroform-methanol 96:4, 2×) purification of the fraction yielded the mentioned cyclodepsipeptides in pure form. The third cyclodepsipeptide was obtained in small amounts (2.6 mg), and identified as desmethyldestruxin B (**18**). TLC: R_f 0.30 (dichloromethane-acetone 70:30), 0.41 (chloroform-methanol 95:5). IR ($CHCl_3$ cast): 3390, 3290, 2960, 1735, 1647, 1520, 1180 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ 8.26 (1H, dd, 2, 8.5 Hz), 7.04 1H, d, 9 Hz), 6.24 (1H, d, 6 Hz), 5.06 (1H, q, 6.5 Hz), 4.89 (1H, dd, 3, 11 Hz), 4.62 (1H, bd, 6.5 Hz), 4.51 (1H, dd, 6.5, 9 Hz), 4.23 (1H, dd, 6, 9.5 Hz), 4.00 (1H, dddd, 13, 10, 5, 2 Hz), 3.91 (1H, bt, 8.5 Hz), 3.44 (1H, bq, 9 Hz), 3.16 (1H, dddd, 13, 11.5, 2, 2 Hz), 2.73 (3H, s), 2.68 (1H, ddd, 18, 11, 2 Hz), 2.60 (1H, ddd, 18, 5, 2 Hz), 2.46 (1H, m), 1.33 (3H, d, 7 Hz), 1.03 (3H, d, 7 Hz), 0.99 (3H, d, 7 Hz), 0.94 (3H, d, 6.5 Hz), 0.92 (3H, d, 7 Hz), 0.90 (3H, t, 7.5 Hz), 0.89 (3H, d, 7.5 Hz). HRMS: m/z calcd. for $C_{29}H_{49}N_5O_7$ (M^+): 579.3632; found: 579.3621(33), 522(58), 494(5), 451(6), 423(9), 70(79), 58(100). CIMS (NH_3): 581(30), 580(100).

Desmethyldestruxin B-methylester (**18'**)

Desmethyldestruxin B (**18**, 0.6 mg), methanol (1 mL) and a catalytic amount of potassium carbonate were allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (10 mL). Dichloromethane extraction (3×5 mL) followed by drying (anh. sodium sulfate) and evaporation of the solvent afforded the crude methanolysis product (0.8 mg). Purification of the crude by dry flash column chromatography (benzene-acetone 70:30, 4 in long, 0.3 cm diameter) yielded 0.6 mg (yield = 95%) of pure desmethyldestruxin B-methylester (**18'**). TLC: R_f 0.45 (dichloromethane-acetone-methanol 70:26:4). IR ($CHCl_3$ cast): 3290, 2960, 1740, 1625, 1539 cm^{-1}. HRMS: m/z calcd. for $C_{30}H_{53}N_5O_8$ (M^+): 611.3894; found: 611.3883(0.4), 580(2), 508(3), 424(33), 325(30), 212(28), 86(16), 70(100).

Isolation of Albrassitriol (**26**, 6α,9α,11-trihydroxydrim-7-ene)

Chromatography fraction 10 (3.5 mg) from the medium polarity crude extract gave a positive bioassay and displayed two components by TLC, one of which was identified as destruxin B (**13**). The second component was purified by dry flash column chromatography (benzene-acetone 80:20, 4 in long, 0.7 cm diameter). Albrassitriol (**26**, 2.6 mg) was obtained as a white amorphous powder, mp

100-104°. TLC: R_f 0.37 (benzene-acetone 70:30), 0.15 (ethyl ether-Skellysolve B 75:25), 0.19 (chloroform-methanol 97.5:2.5). IR ($CHCl_3$ cast): 3299, 2922, 1450, 1006 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$):δ5.52 (1H, bd, 1 Hz), 4.17 (1H, dd, 1, 10 Hz), 3.78 (1H, d, 11 Hz), 3.60 (1H, dd, 1, 10 Hz), 2.52 (1H, bs), 2.09 (1H, bs), 1.84 (3H, bdd, 1.5 Hz), 1.72 (1H, dt, 3.5, 11.5 Hz), 1.63 (1H, d, 10 Hz), 1.18 (3H, s), 1.08 (3H, s), 0.88 (3H, s). ^{13}C NMR ($C\underline{D}Cl_3$): δ131.5 (s), 123.6 (d), 76.7 (s), 68.7 (d), 62.0 (t), 50.4 (d), 42.9 (t), 42.7 (t), 38.0 (s), 33.3 (s), 32.6 (q), 22.8 (q), 19.6 (q), 18.6 (t), 16.8 (q). HRMS: m/z calcd. for $C_{15}H_{26}O_3$ (M^+): 254.1882; found: 254.1876(3), 236(16), 223(75), 205(27), 187(14), 154(10), 137(11), 130(100), 112(63). CIMS (NH_3): 272(51), 254(100), 237(54), 219(94), 201(48).

O,O-Diacetylalbrassitriol (**D'**, 6α,11-diacetoxy-9α-hydroxydrim-7-ene

A mixture of albrassitriol (**26**, 2.6 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) was allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (20 mL), extracted with ethyl acetate (3×10 mL) and worked up in the usual manner to afford 3.6 mg of crude product. Purification of the acetylated product by dry flash column chromatography (benzene-acetone 97:3, 4 in long, 0.7 cm diameter) yielded

O,O-diacetylalbrassitriol (**D'**, 1.2 mg, yield = 35%). TLC: R_f 0.32 (benzene-acetone 95:5), 0.50 (benzene-acetone 90:10), 0.43 (chloroform-methanol 98:2). IR ($CHCl_3$ cast): 3480, 2930, 1733, 1372 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ5.44 (1H, dd, 1.5, 1.5 Hz), 5.37 (1H, ddd, 2, 2, 10 Hz), 4.26 (1H, d, 12 Hz), 4.21 (1H, d, 12 Hz), 2.36 (1H, s), 2.12 (1H, d, 10 Hz), 2.08 (3H, s), 2.05 (3H, s), 1.77 (3H, dd, 1.5, 1.5 Hz), 1.01 (6H, s), 0.98 (3H, s). ^{13}C NMR ($C\underline{D}Cl_3$): δ170.7 (2×, s), 137.6 (s), 127.5 (d), 75.0 (s), 71.8 (d), 64.8 (t), 46.0 (t), 43.5 (d), 42.7 (t), 35.6 (s), 33.2 (s), 32.9 (q), 23.1 (q), 21.8 (q), 21.0 (q), 19.3 (q), 18.6 (q), 17.6 (t). HRMS: m/z calcd. for $C_{17}H_{26}O_3$ (P - $C_2H_3O_2$, parent ion peak not seen): 278.1882; found: 278.1886(87), 236(13), 218(85), 214(27), 205(82), 200(3), 154(19), 137(10), 112(100). CIMS (NH_3): 356(1), 296(26), 280(20), 279(100), 219(16), 201(54).

Albrassitriol-acetonide (**D"**, 9α,11-O-isopropylidine-6α-hydroxydrim-7-ene

Albrassitriol (**26**, 0.8 mg), 2,2-dimethoxypropane (1.5 mL), and p-toluenesulfonic acid (catalytic amount) were allowed to stir at room temperature. The course of the reaction was monitored by TLC, and after two hours no starting material was observed. The reaction mixture was diluted with ethyl ether (30 mL) and washed with saturated

$NaHCO_3$ (3×10 mL). The organic extract was dried (anh. sodium sulfate) and concentrated to give a yellowish oil (1 mg). Purification by means of dry flash column chromatography (benzene-acetone 96:4, 4 in long, 0.4 cm diameter) afforded albrassitriol-acetonide (**D"**, 0.4 mg, yield = 38%) as a colorless oil. TLC: R_f 0.45 (benzene-acetone 90:10), 0.23 (chloroform-methanol 98:2). IR ($CHCl_3$ cast): 3440, 2921, 1450, 1380, 1260, 1061 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ5.56 (1H, bs), 4.10 (1H, bs), 3.89 (2H, q, 10 Hz), 2.17 (1H, d, 2 Hz), 1.86 (3H, dd, 2, 2 Hz), 1.59 (1H, d, 10 Hz), 1.49 (3H, s), 1.39 (3H, s), 1.17 (3H, s), 1.05 (3H, s), 0.83 (3H, s). HRMS: m/z calcd. for $C_{18}H_{30}O_3$ (M^+): 294.2195; found: 294.2190(29), 279(16), 236(7), 219(4), 201(4), 170(30), 137(5), 112(100). CIMS (NH_3): 312(7), 294(14), 278(21), 277(100), 219(12).

Isolation of isoalbrassitriol (**28**, 8β,9α,11-trihydroxydrim-6-ene)

Chromatography fraction 7 (2.8 mg) of the medium polarity crude extract was further purified by dry flash column chromatography (benzene-acetone 90:10, 4 in long, 0.7 cm diameter). This afforded isoalbrassitriol (**28**, 0.7 mg) as a clear oil. TLC: R_f 0.55 (benzene-acetone 70:30), 0.59 (dichloromethane-acetone 70:30). IR ($CHCl_3$ cast): 3400, 2926, 1458, 1383, 1260, 1074 cm^{-1}. ^1H NMR

(400 MHz, $C\underline{D}Cl_3$): δ5.77 (1H, dd, 3, 10 Hz), 5.55 (1H, dd, 3, 10 Hz), 4.11 (1H, d, 12 Hz), 3.86 (1H, d, 12 Hz), 3.87 (1H, bs), 2.37 (1H, t, 3 Hz), 2.00 (1H, d, 2 Hz), 1.37 (3H, s), 1.17 (1H, d, 10 Hz), 1.08 (3H, s), 0.96 (3H, s), 0.89 (3H, s). HRMS: m/z calcd. for $C_{15}H_{26}O_3$ (M^+): 254.1882; found: 254.1877(6), 236(9), 221(10), 218(6), 205(35), 193(48), 175(34), 137(10), 125(100), 107(28). CIMS (NH_3): 272(4), 255(6), 254(26), 253(25), 237(72), 219(100), 201(47).

O-Acetylisoalbrassitriol (**G'**, 11-acetoxy-8β-9α-dihydroxydrim-6-ene)

A mixture of isoalbrassitriol (**28**, 0.9 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) was allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (10 mL), extracted with ethyl acetate (3× 10 mL), and worked up in the usual manner. Dry flash column chromatography (benzene-acetone 96:4, 4 in long, 0.3 cm diameter) purification of the crude acetylated product (1.1 mg) afforded O-acetylisoalbrassitriol (**G'**, 0.5 mg, yield = 48%). TLC: R_f 0.36 (benzene-acetone 90:10), 0.33 (chloroform-methanol 98:2). IR ($CHCl_3$ cast): 3520, 2927, 1730, 1460, 1383, 1238, 1039 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ5.76 (1H, dd, 2, 10 Hz), 5.53 (1H, dd, 3, 10 Hz), 4.54 (1H, d, 12 Hz), 4.33 (1H, d, 12 Hz), 2.49 (1H, s), 2.40 (1H, bt, 2 Hz), 2.13 (3H, s), 1.37 (3H, s), 1.08 (3H, s), 0.96 (3H, s), 0.88 (3H, s). HRMS: m/z calcd. for $C_{17}H_{28}O_4$ (M^+): 296.1988; found: 296.1964(3), 278(3), 236(37), 218(13), 205(41), 200(3), 193(40), 175(53), 137(15), 125(100), 107(28). CIMS (NH_3): 314(20), 296(38), 279(100), 219(14), 201(29).

Reaction of O-Acetylisoalbrassitriol (**G'**) with 2,2-dimethoxypropane

O-acetylisoalbrassitriol (**G'**, 0.3 mg) was dissolved in 2,2-dimethoxypropane (1 mL) and a catalytic amount of p-toluenesulfonic acid added. The solution was allowed to stir at room temperature and the course of the reaction was monitored by TLC. After 48 h of reaction no significant change was observed. The reaction mixture was diluted with ethyl ether (30 mL), and the organic layer washed with saturated $NaHCO_3$ (3×10 mL). Concentration of the extract yielded a crude product which showed O-acetylisoalbrassitrol (**G'**) as the major component on TLC.

Isolation of deoxyuvidin B (**30**, 3β,11-dihydroxy-6-oxo-drim-7-ene)

Deoxyuvidin B (**30**) was isolated (1.6 mg) as a white powder by the preparative TLC (20×20 cm, chloroform-methanol 95:5, 2×) purification of fraction 12 (39.8 mg). TLC: R_f 0.24 (chloroform-methanol, 95:5), 0.29

(benzene-acetone 70:30). IR (CHCl$_3$ cast): 3440, 2925, 1652, 1440, 1383, 1237, 1047 cm^{-1}. UV (CH$_3$OH) λ_{max}: 235 nm (ε 1763). ^{1}H NMR (400 MHz, CDCl$_3$): δ5.86 (1H, bd, 0.5 Hz), 3.97 (1H, dd, 3.5, 11 Hz), 3.86 (1H, dd, 6, 11 Hz), 3.22 (1H, dd, 4.5, 10.5 Hz), 2.29 (1H, bs), 2.13 (1H, bs), 2.05 (3H, bd, 0.5 Hz), 1.27 (3H, s), 1.16 (3H, s), 0.92 (3H, s). HRMS: m/z calcd. for $C_{15}H_{24}O_3$ (M$^+$): 252.1725; found: 252.1725(18), 234(17), 221(3), 216(17), 203(4), 149(10), 123(100), 81(17). CIMS (NH$_3$): 270(20), 254(21), 253(100), 252(5).

O,O-Diacetyldeoxyuvidin B (**B'**, 3β,11-diacetoxy-6-oxo-drim-7-ene

Deoxyuvidin B (**30**, 1.1 mg) was treated with acetic anhydride (1 mL) and pyridine (0.5 mL), and allowed to stir overnight at room temperature. The reaction mixture was poured over water-ice (20 mL) and extracted with ethyl acetate (3×10 mL). Work up of the organic layer in the usual fashion afforded the crude acetylated product (1.8 mg). Purification by preparative TLC (10×10 cm, benzene-acetone 95:5, 2×) yielded pure O,O-diacetyldeoxyuvidin B (**B'**, 0.4 mg, yield = 27%). TLC: R$_f$ 0.51 (benzene-acetone 90:10), 0.34 (Skellysolve B-acetone 70:30), 0.3 (Skellysolve B-ethyl acetate 75:25). IR (CHCl$_3$ cast): 2920, 1738, 1671, 1366, 1241 cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$): δ5.87 (1H, bd, 1 Hz), 4.45 (1H, dd, 4.5, 10 Hz), 4.33 (1H, dd, 3, 12 Hz), 4.22 (1H, dd, 6, 12 Hz), 2.49 (1H, bs), 2.20 (1H, s), 2.07 (6H, s), 1.95 (1H, bs), 1.23 (3H, s), 1.15 (3H, s), 0.96 (3H, s). HRMS: m/z calcd. for $C_{17}H_{24}O_3$ (P - $C_2H_3O_2$, parent ion not seen): 276.1725; found: 276.1727(20), 234(2), 216(28), 175(22), 135(100), 112(38), 81(8). CIMS (NH_3): 673(2), 354(34), 338(24), 337(100), 335(30), 278(15), 277(76), 217(21), 216(27).

Epoxidation of deoxyuvidin B (**30**)

Deoxyuvidin B (**30**, 0.3 mg) was dissolved in methanol (0.5 mL) containing 30% hydrogen peroxide (1 drop). The solution was cooled to 20° and 6N sodium hydroxide (2 drops) was added. The reaction mixture was stirred overnight at room temperature. After 18 h the TLC showed that all of the starting material had disappeared. The mixture was diluted with water (10 mL) and the resulting aqueous suspension extracted with ethyl ether (3×5 mL). The organic extract was washed with saturated NaCl (10 mL), dried (anh. sodium sulfate), and concentrated to give crude product (0.4 mg). IR (CH_2Cl_2 cast): 3400, 1700, 1667 cm^{-1}. HRMS: 234(48), 220(2), 219(18), 216(7), 167(5), 149(8), 123(5).

The crude reaction product presented two major components by TLC. The least polar component was UV active while the other component presented a similar R_f value to that of the starting material but was not visible under UV light. Purification of the mixture by preparative TLC (5×7.5 cm, chloroform-methanol 95:5, 2×) provided each component. The data of the less polar component (0.1 mg) seem to indicate that it is actually a mixture of two compounds. This mixture was not further resolved. TLC: R_f 0.63 (benzene-acetone 70:30), 0.50 (chloroform-methanol 95:5). IR (CH_2Cl_2 cast): 3430, 1727, 1668, 1465, 1381, 1042 cm^{-1}. HRMS: 234(13), 216(3), 149(3).

The more polar compound (0.2 mg) was identified as uvidin B (**34**) on the basis of its TLC behavior and spectroscopic data by direct comparison with an authentic sample. TLC: R_f 0.24 (benzene-acetone 70:30), 0.26 (chloroform-methanol 95:5), 0.15 (benzene-acetone 90:10). IR (CH_2Cl_2 cast): 3400, 2920, 1720, 1460, 1380, 1275, 1070 cm^{-1}. HRMS: m/z calcd. for $C_{15}H_{24}O_4$ (M^+): 268.1674; found: 268.1656(1), 222(1), 167(41), 149(11), 123(7).

Isolation of brassicadiol (**38**, 2,3-dihydro-6-propyl-2-[2-hydroxypropyl]-7-hydroxymethyl benzofuran)

Purification of chromatography fraction 9 (10.7 mg) of the medium polarity crude extract by dry flash column chromatography (benzene-acetone 90:10, 4 in long, 0.8 cm diameter) gave brassicadiol (**38**, 2.7 mg) as a milky-white oil. TLC: R_f 0.43 (benzene-acetone 70:30), 0.37

(chloroform-methanol 96:4), 0.33 (ethyl ether-Skellysolve B 75:25). IR (CH_2Cl_2 cast): 3335, 2957, 2928, 1590, 1442, 1380 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ 7.02 (1H, d, 7.5 Hz), 6.69 (1H, d, 7.5 Hz), 4.73 (2H, s), 4.64 (1H, dd, 9, 9 Hz), 3.17 (1H, dd, 9, 15.5 Hz), 3.11 (1H, dd, 9, 15.5 Hz), 2.62 (2H, bt, 7.5 Hz), 2.16 (1H, m), 2.04 (1H, bs), 1.58 (2H, ddd, 8, 8, 9.5 Hz), 1.28 (3H, s), 1.14 (3H, s), 0.97 (3H, t, 7.5 Hz). ^{13}C NMR ($C\underline{D}Cl_3$): 158.5 (s), 141.0 (s), 124.7 (s), 124.1 (d), 122.0 (d), 120.6 (s), 89.9 (d). 72.0 (s), 57.5 (t), 34.7 (t), 30.6 (t), 26.3 (q), 25.1 (t), 24.3 (q), 14.1 (q). HRMS: m/z calcd. for $C_{15}H_{22}O_3$ (M^+): 250.1569; found: 250.1565(24), 192(2), 174(100), 131(13), 59(25). CIMS (NH_3): 501(5), 500(16), 269(14), 268(75), 251(18), 250(100), 233(16).

O-Acetylbrassicadiol (**I'**, 2,3-dihydro-6-propyl-2-[2-hydroxypropyl]-7-acetoxymethyl benzofuran)

Brassicadiol (**38**, 0.9 mg) was allowed to stir overnight at room temperature with acetic anhydride (1 mL) and pyridine (0.5 mL). The reaction mixture was poured over water-ice (10 mL), extracted with ethyl acetate (3×10 mL), and worked up in the usual way to give crude acetylated product (1.9 mg). The major, most polar component was purified by dry flash column chromatography (benzene-acetone 96:4, 4 in long, 0.7 cm diameter) and identified as O-acetylbrassicadiol (**I'**, 0.6 mg, yield =

57%). TLC: R_f 0.37 (benzene-acetone 90:10), 0.36 (ethyl ether-Skellysolve B 3:2), 0.17 (benzene-acetone 96:4). IR ($CHCl_3$ cast): 3480, 2960, 2928, 1736, 1590, 1445, 1380, 1237, 1020 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ7.06 (1H, ddd, 1, 1, 7.6 Hz), 6.69 (1H, bd, 7.6 Hz), 5.27 (1H, d, 11.6 Hz), 5.08 (1H, d, 11.8 Hz), 4.62 (1H, dd, 9, 9 Hz), 3.15 (2H, bd, 9 Hz), 2.62 (1H, bdd, 8, 14 Hz), 2.54 (1H, bdd, 8, 14 Hz), 2.16 (1H, bs), 2.08 (3H, s), 1.60 (2H, m), 1.38 (3H, s), 1.22 (3H, s), 1.00 (3H, t, 7 Hz). HRMS: m/z calcd. for $C_{17}H_{23}O_4$ (M^+): 292.1674; found: 292.1673(12), 217(7), 174(100), 131(9), 59(34). CIMS (NH_3): 542(19), 311(20), 310(100), 292(2), 250(49), 231(26).

Oxidation of O-Acetylbrassicadiol (I')

O-acetylbrassicadiol (I', 0.4 mg) was dissolved in acetone (2 mL) and Jones reagent (0.3 mL) was added. The mixture was allowed to stir at room temperature. After 90 min the reaction was diluted with ethyl ether (3×5 mL). The ether extract was dried and concentrated. The reaction product showed starting material as the only component.

Appendix of Experimental

Modified Czapek-Dox medium

100 g sucrose

2 g casein hydrolysate

1.5 g $NaNO_3$

1.5 g $NaNO_3$

1.0 g K_2HPO_4

0.5 g KCl

0.5 g $MgSO_4$

0.01 g $FeSO_4$

Complete to 1 L with water and adjust pH to 6.0.

V-8 juice medium

200 mL V-8 juice (unfiltered)

0.75 g $CaCO_3$

0.05 g rose bengal (optional)

water to 1 L.

V-8 agar

200 mL V-8 juice (unfiltered)

0.050 g rose bengal

20 g agar

800 mL water

3 g $CaCO_3$.

Phosphomolybdic acid reagent for TLC

20 g Phosphomolybdic acid (20 $MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$)

2.5 g Ceric sulfate ($H_4Ce(SO_4)_4$)

Dissolve in 500 mL of 5% aqueous H_2SO_4.

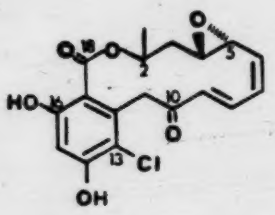
STUDY II

INTRODUCTION

Monocillium nordinii (Bourchier) W. Gams is a destructive mycoparasite of the pine stem rusts Cronartium coleosporoides Arth. and Endocranartium harknessii (J.P. Moore) Y. Hiratsuka.[1] Outbreaks of these rusts in western Canada cause severe damage to hard pines.

Previous work in these laboratories[2] has shown that two of the metabolites produced by M. nordinii are active against pine stem rusts as well as other fungi, including Ceratocystin ulmi the fungus responsible for "dutch elm" disease. The two active components have been identified as monorden (**1**) and monocillin I (**2**) Monorden (**1**) first isolated in 1953 by Delmotte from Monosporium bonorden[3,4] is a compound with antibiotic properties also found in culture extracts from nectria radicicola[5,6] and other fungi.[7,8] The structure of monorden (**1**) was first determined in 1964, and reported separately by Delmotte[4] and Mirrington.[5,6] Monocillin I (**2**) is a new natural product first isolated in these laboratories.[2]

Other new compounds isolated and identified from the same culture filtrates of Monocillium nordinii included monocillins II (**3**), III (**4**), IV (**5**), and V (**6**).[2] Purification of the mycelium extract afforded monorden



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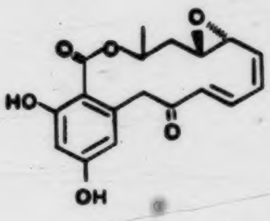

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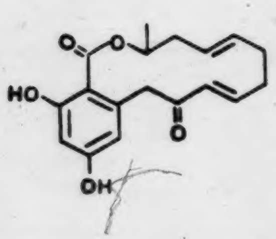

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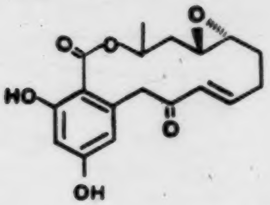

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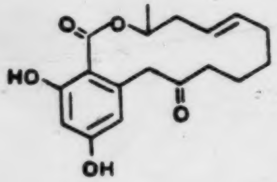

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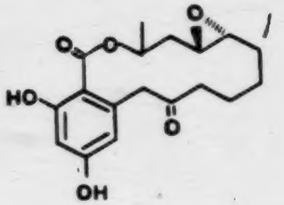

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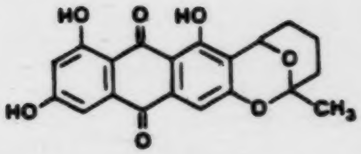
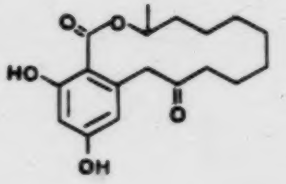


7

and the monocillins in addition to Averufin (**7**) and an unidentified yellow pigment.[2]

Our work with Monocillium nordinii was divided into three parts. In part one the purification of the broth extract was repeated in order to obtain significant amounts of the previously reported metabolites, primarily monocillin I, for use in chemical correlation studies (part two). During this study two new natural products, nordinone (**8**) and nordinonediol (**9**) were obtained along with the aforementioned metabolites (with the exception of monocillin V).

In addition, the low-molecular weight compounds uracil (**10**) and succinic acid (**11**) were isolated. Purification of the mycelial extract yielded the previously reported yellow pigment, but no trace of Averufin was found. The yellow pigment was identified as sterigmatocystin (**12**) by direct comparison with an authentic sample. The common fungal metabolite ergosterol endoperoxide (**13**) was also obtained from the mycelial extract.



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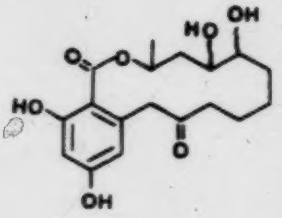

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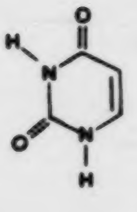

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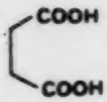

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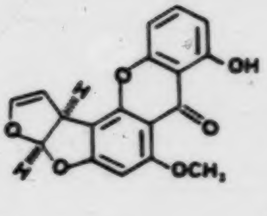

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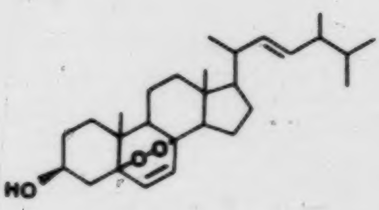

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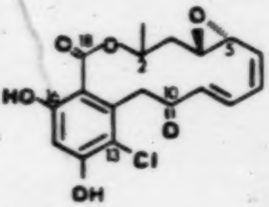
Included in the second part are studies on the chlorination and bromination of monocillin I (**2**). The last part reports a study of the growth of *Monocillium nordinii* under different culture conditions in order to observe the variations in the production of Monorden and monocillin I.

RESULTS AND DISCUSSION

The fungus Monocillium nordinii is a destructive mycoparasite of pine stem rusts. It has been reported to produce the metabolites monorden (**1**) and monocillin I (**2**).[2] Both compounds display significant antifungal activity against a wide range of fungi including Ceratocystis ulmi, the cause for "Dutch elm" disease. This study of the chemistry of M. nordinii will be described in three parts, the first dealing with isolation and structural studies, the second with chemical modification of the metabolites, and the third with the culturing of the fungus.



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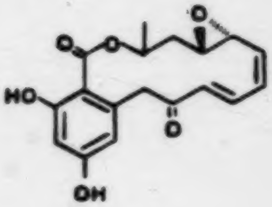
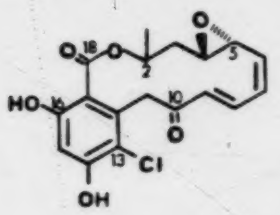


2

Part I. Isolation and Identification of Metabolites

Chemical investigations of the metabolites produced by M. nordinii when it is grown in still culture have been reported previously from these laboratories.[2] The early work describes the isolation and identification of monorden (**1**), five new compounds known as the monocillins (I, II, III, IV, and V; **2**, 3, **4**, 5, and 6, respectively), averufin (**7**), and an unidentified yellow pigment, $C_{18}H_{12}O_6$.



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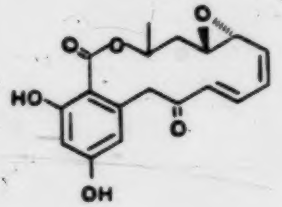

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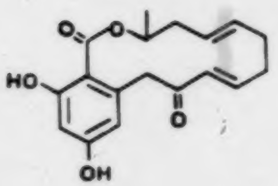

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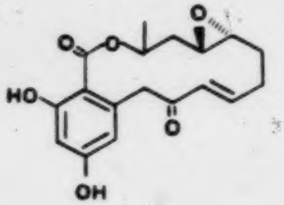

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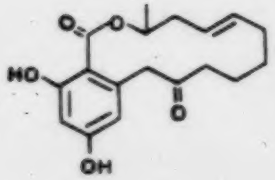

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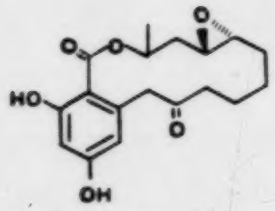

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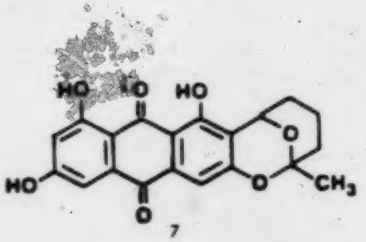

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Further chemical investigations of the metabolites produced by M. nordinii were undertaken for several reasons: to obtain larger samples of the known metabolites for chemical and biological studies, to isolate and identify new metabolites, and to re-isolate and identify the yellow pigment. Monocillium nordinii was grown in still culture in Difco potato dextrose broth containing 0.2% yeast extract for 14 days. The broth was separated from the mycelium and the broth and mycelium were separately extracted in the manner previously described.[2]

The mycelial extract was subjected to chromatographic separation over silica gel. Chromatography fractions containing the yellow pigment were combined and purified by preparative TLC. The yellow pigment, compound **A**, was isolated as an oil. Compound **A** has a molecular weight of 324 corresponding to a molecular formula $C_{18}H_{12}O_6$ as shown by HRMS.

The IR spectrum of **A** shows hydrogen-bonded hydroxyl absorption at 3200 cm^{-1}. Its ^1H NMR spectrum (Figure 1) indicates the presence of four aromatic protons (δ7.48, 6.77, 6.70, 6.40), a methoxyl group (δ3.98), and a low field D_2O-exchangeable signal attributed to the hydrogen of an internally hydrogen-bonded phenolic hydroxyl (δ13.25). These properties and molecular formula

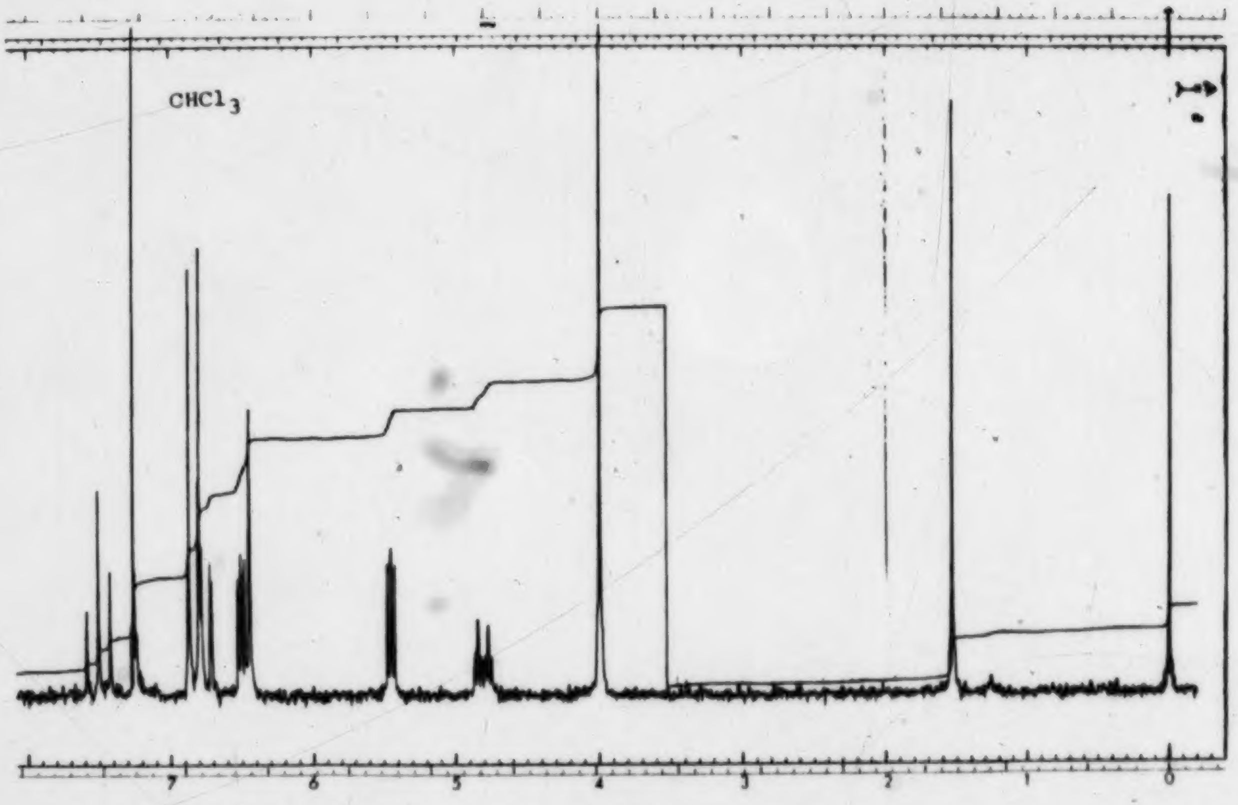
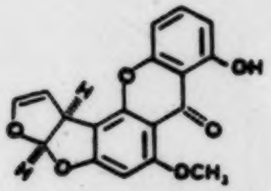


Figure 1. 100 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of sterigmatocystin (8).

suggested that compound **A** may be the known carcinogenic mycotoxin sterigmatocystin[9,10] (**8**).



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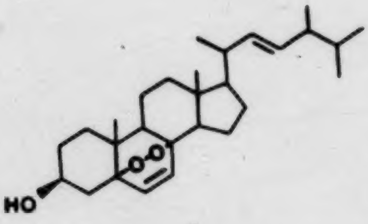
Comparison of **A** with a commercially available sample of sterigmatocystin (**8**) showed that they were identical (TLC and superimposable IR and ^{1}H NMR).

Another metabolite isolated from the chromatographic fractions of the mycelial extract of M. nordinii was compound **B**. Compound **B** was identified as the ubiquitous ergosterol endoperoxide, **9**, a fungal metabolite isolated from a host of fungal sources. Compound **9** shows hydroxyl (3420 cm^{-1}) as well as C-O (1100 and 1040 cm^{-1}) stretching bands in its IR spectrum. The HRMS of **9** shows a molecular

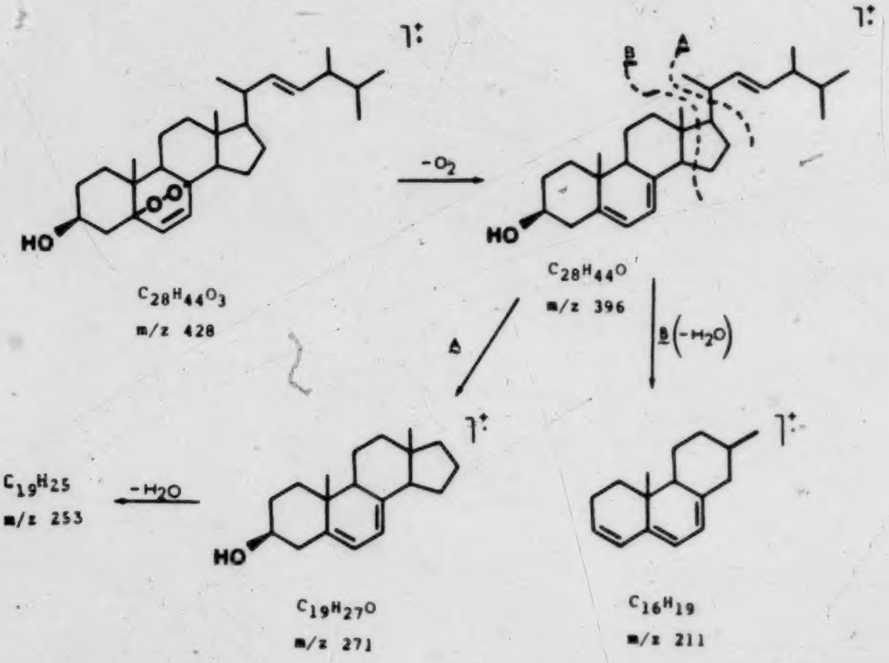


9

ion at m/z 428 ($C_{28}H_{44}O_3$) and the characteristic fragmentation[11,12,13] pattern as outlined in Scheme I.

Scheme I

MS Fragmentation of Ergosterol Endoperoxide (**9**)



HO
$C_{28}H_{44}O_3$
m/z 428
$-O_2$
HO
$C_{28}H_{44}O$
m/z 396
A
B
A
B ($-H_2O$)
$C_{19}H_{25}$
m/z 253
$-H_2O$
HO
$C_{19}H_{27}O$
m/z 271
$C_{16}H_{19}$
m/z 211

The ^1H NMR spectrum of ergosterol endoperoxide (**9**, Figure 2) reveals the presence of a carbinol proton (δ3.84, m) and four vinylic protons (δ6.48, 6.22; doublet each, J = 6

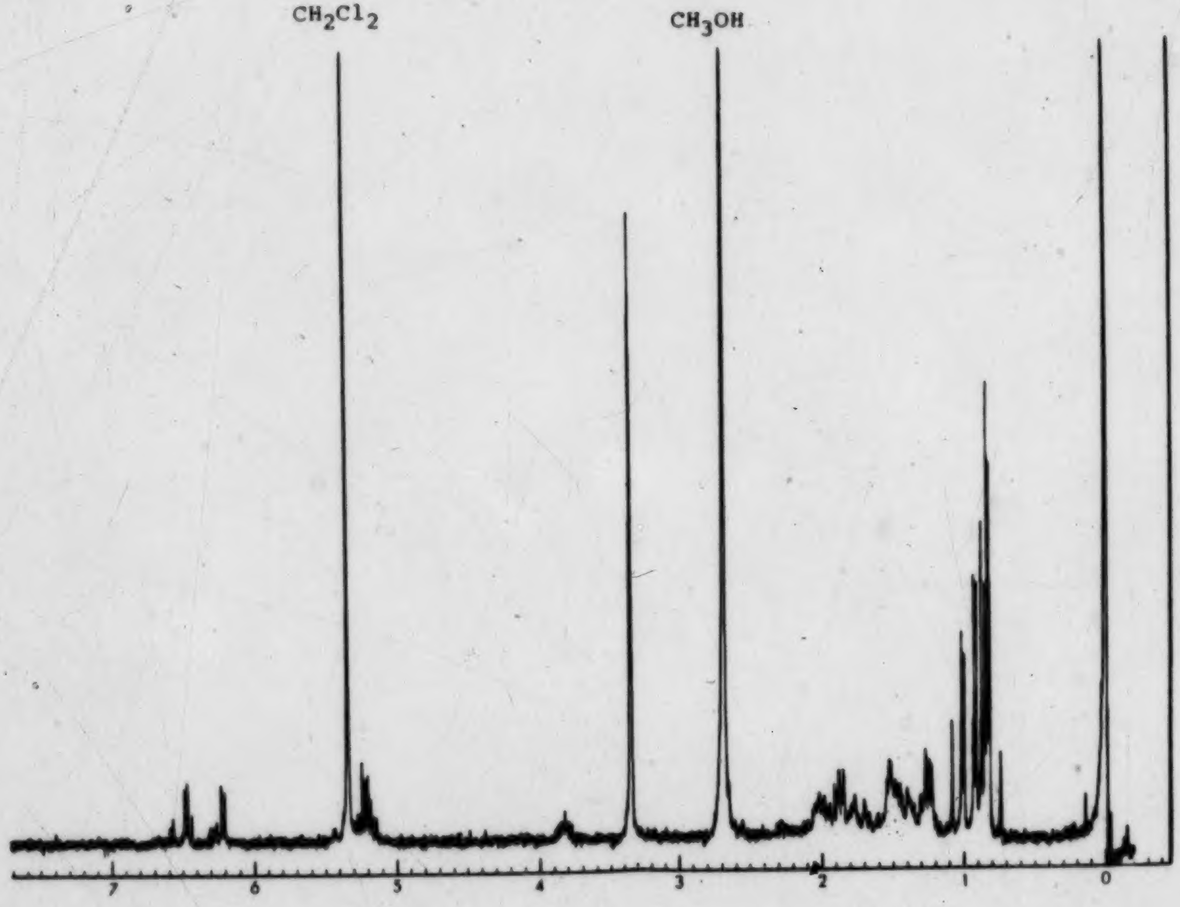


Figure 2. 400 MHz ^1H NMR spectrum ($C\underline{D}_2Cl_2/C\underline{D}_3O\underline{D}$) of ergosterol endoperoxide (9).

Hz; δ5.22, m).

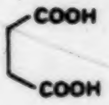
Confirmation that compound **B** was indeed ergosterol endoperoxide (**9**) was achieved by comparison of the spectral properties of **B** with those of an authentic sample of ergosterol endoperoxide (**9**).

Further investigation of the minor metabolites of the mycelium extract of *M. nordinii* was discontinued since we did not want to risk further possible exposure to the highly carcinogenic sterigmatocystin (**8**).

Isolation of the metabolites from the broth of *M. nordinii* which do not include sterigmatocystin as indicated by TLC, was attempted following the previous procedure.[2] This procedure involves column chromatographic purification of the crude broth extract using a gradient elution (chloroform-methanol). However, this method presented several disadvantages; the procedure is time consuming and very often the metabolites are contaminated with pigments which are difficult to separate.

Removal of the contaminating pigments was initially attempted using reverse-phase column chromatography. The broth extract was eluted through a Sephadex LH-20 column using methanol as the eluent. The problem of the pigment contamination was not solved in this manner. However, two crystalline and very polar compounds, **C** and **D**, were isolated and identified.

Compound **C** was identified as succinic acid (**10**) on the basis of its spectroscopic data. Succinic acid (**10**)

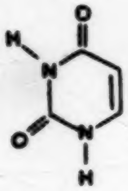


10

has a molecular formula $C_6H_6O_4$ (mol. wt. 118), and its ^1H NMR shows only two singlets, in a 2:1 ratio, at high (δ2.40) and low field (δ10.92). Identification of compound **C** as succinic acid (**10**) was complete when the IR spectrum of **C** was shown to be superimposable on that of an authentic sample of succinic acid (**10**).

The second crystalline compound, **D**, has a very high melting point (>350°) and its formula was found by HRMS to be $C_4H_4N_2O_2$ (mol. wt. 312). A N-H stretching vibration is observed at 3400 cm^{-1} in the IR spectrum of **D**. This value is typical of that observed for secondary amides.[14] Amide I bands are present at 1730 and 1716 cm^{-1},[14,15] while the amide II bands appear, along with other absorptions, in the region between 1680-1640 cm^{-1}.[14,15] The ^1H NMR of compound **D** shows two N-H protons as a broad singlet at δ11.06, and two vinylic protons as doublets (each J = 8 Hz) at δ7.40 and 5.45.

These data allow the tentative identification of **D** as uracil (**11**). Confirmation of the structure was attained by comparison of the IR spectrum of **D** with that of an authentic sample of uracil (**11**).



11

The problem of pigment contamination was overcome by using the "dry flash" column chromatography technique.* This chromatographic procedure is fast, reliable, and usually leads to good purification. Using this chromatographic method the order of elution of the metabolites is somewhat different to that observed previously.[2] The first metabolite to be eluted is monocillin IV (**5**), which is followed by monocillin II (**3**), monocillin I (**2**), monorden (**1**), and monocillin III (**4**). This order of elution is also observed when the metabolites are subjected to TLC using ethyl ether-Skellysolve B (3:1).

---

*For complete description see Experimental.

Dry flash chromatographic separation of the broth extract gave compound **E** as colorless needles, mp 175°-178°. Compound **E** has a molecular formula of $C_{18}H_{17}O_6Cl$ (mol. wt. 366; six unsaturations). The presence of chlorine in the structure of **E** was demonstrated in its HRMS where the ratio of relative intensity of the molecular ion at m/z 366 to that corresponding to $M^+ -2$ (m/z 364) is 1:3.[16]

The IR spectrum of compound **E** presents a broad band at 3300 cm^{-1} suggesting the presence of a hydrogen-bonded hydroxyl group. Other bands include one at 1655 cm^{-1} characteristic of an α,β-unsaturated carbonyl group, one at 1630 cm^{-1} typical of an *ortho*-CO-C_6H_4-OH system,[15] and others in the 1620-1600 cm^{-1} region assigned to aromatic and/or double bond functionalities.[14]

The HRMS of **E** is dominated by intense peaks, in a ratio of 1:3,[16] at m/z 186 ($C_8H_5O_3{}^{37}Cl$) and m/z 184 ($C_8H_5O_3{}^{35}Cl$). The number of unsaturations (six) implied by the formula of the fragment ($C_8H_5O_3Cl$) suggests the presence of an aromatic ring in this ion. The aromatic nature of **E** is supported by the presence in its HRMS of characteristic fragment ions at m/z 65, 77, 78, 79, 91.[14]

The ^1H NMR of **E** (Figure 3) shows a single aromatic proton at δ 6.65 indicating a pentasubstituted aromatic ring. On the basis of this and other (*vide infra*) data it

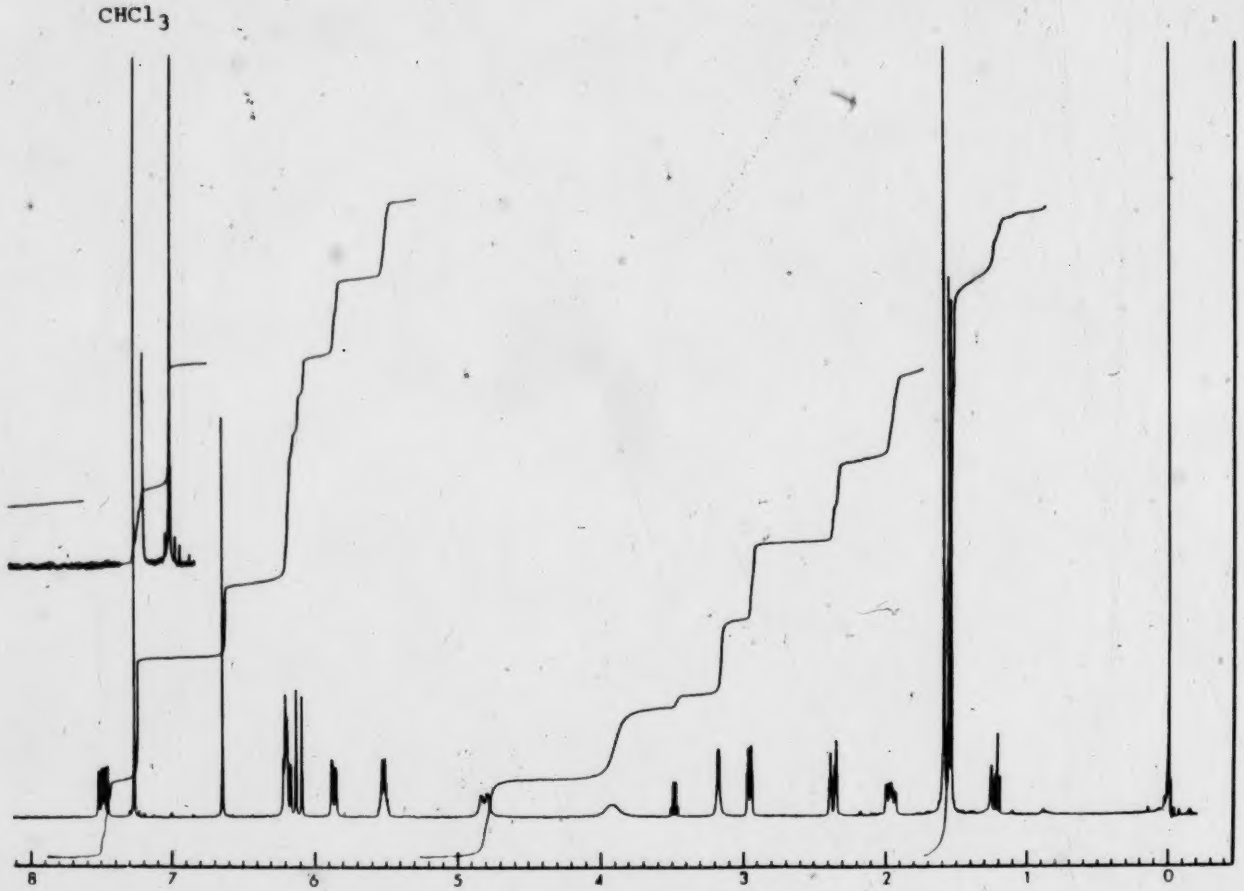
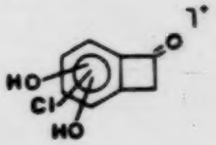


Figure 3. 400 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of monorden (1).

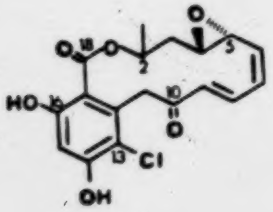
seemed probable that chlorine is one of the aromatic substituents. Thus structure **12** can be proposed for the $C_8H_5O_3Cl$ fragment (m/z 186, 184).



12

Also present in the ^1H NMR of **E** (Figure 3) are one proton singlets at δ6.20 and 11.00, corresponding to hydroxyl groups (D_2O-exchangeable), with the one at lowest field being characteristic of a hydrogen-bonded phenol. One proton signals at δ7.49 (dd, 10, 16 Hz), 6.19 (dd, 11, 11 Hz), 6.11 (d, 16 Hz), and 5.83 (dd, 3, 11 Hz) indicate the presence of four vinylic protons. Three protons geminal to oxygen are observed at δ2.96 (dt, 3, 9 Hz), 3.18 (bs) and 5.51 (m). The nonequivalent protons of a benzylic methylene appear as broad doublets (each J = 16 Hz) at δ4.81 and 3.92. A methyl doublet (J = 7 Hz) is observed at δ1.54.

The preceding data suggested that compound **E** is the known antifungal metabolite monorden (**1**).[3-8] This was confirmed by comparison of **E** with an authentic sample of monorden (**1**).



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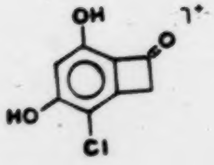
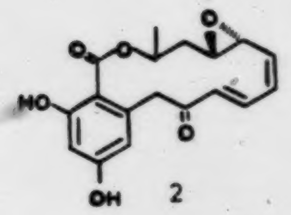


12a

The structure of monorden (**1**) explains the mass spectral fragmentation pattern observed. Fragments of the type shown in **10** (revised to **12a**) are consistent with the known fragmentation of o-hydroxyaromatic esters.[4]

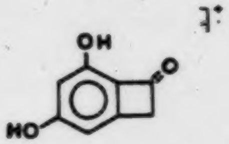
Monorden (**1**) was first isolated as a metabolite of Monosporiun bonorden.[3,4] It has also been isolated from Nectria radicicola,[5,6] Penicilliun luteo-aurantium,[7] and Diheterospora chlanydosporia.[8]

Compound **F** was obtained as an amorphous white solid (mp 75-77°) by recrystallization (ethyl ether-Skellysolve B) of a chromatographic fraction. Compound **F** was identified as monocillin I (**2**), previously isolated as a colorless oil.[2]



2

Monocillin I (**2**) has a molecular formula $C_{18}H_{18}O_6$ (mol. wt. 330), and a ^{1}H NMR spectrum (Figure **4**) very similar to that of monorden (**1**, Figure 3). The base peak at m/z 150 ($C_6H_6O_3$) in the HRMS of monocillin I (**2**) corresponds to the fragment ion **13**).



13

Compound G was obtained in pure form as white crystals, mp 205-207°. The molecular formula of **G**, $C_{18}H_{20}O_6$ (mol. wt. 332), indicates that it has one less unsaturation than monocillin I (**2**). Its IR is very similar to that of **2** indicating the presence of the α,β-unsaturated carbonyl group (1645 cm^{-1}) and the hydrogen bonded phenol (3400 cm^{-1}).

The ^{1}H NMR of compound **G** (Figure 5), unlike that of monocillin I (**2**), shows only two vinylic protons (δ 6.82 and 6.06). These spectroscopic data suggest that **G** is monocillin III (**4**). Comparison of the data of **G** and **4**

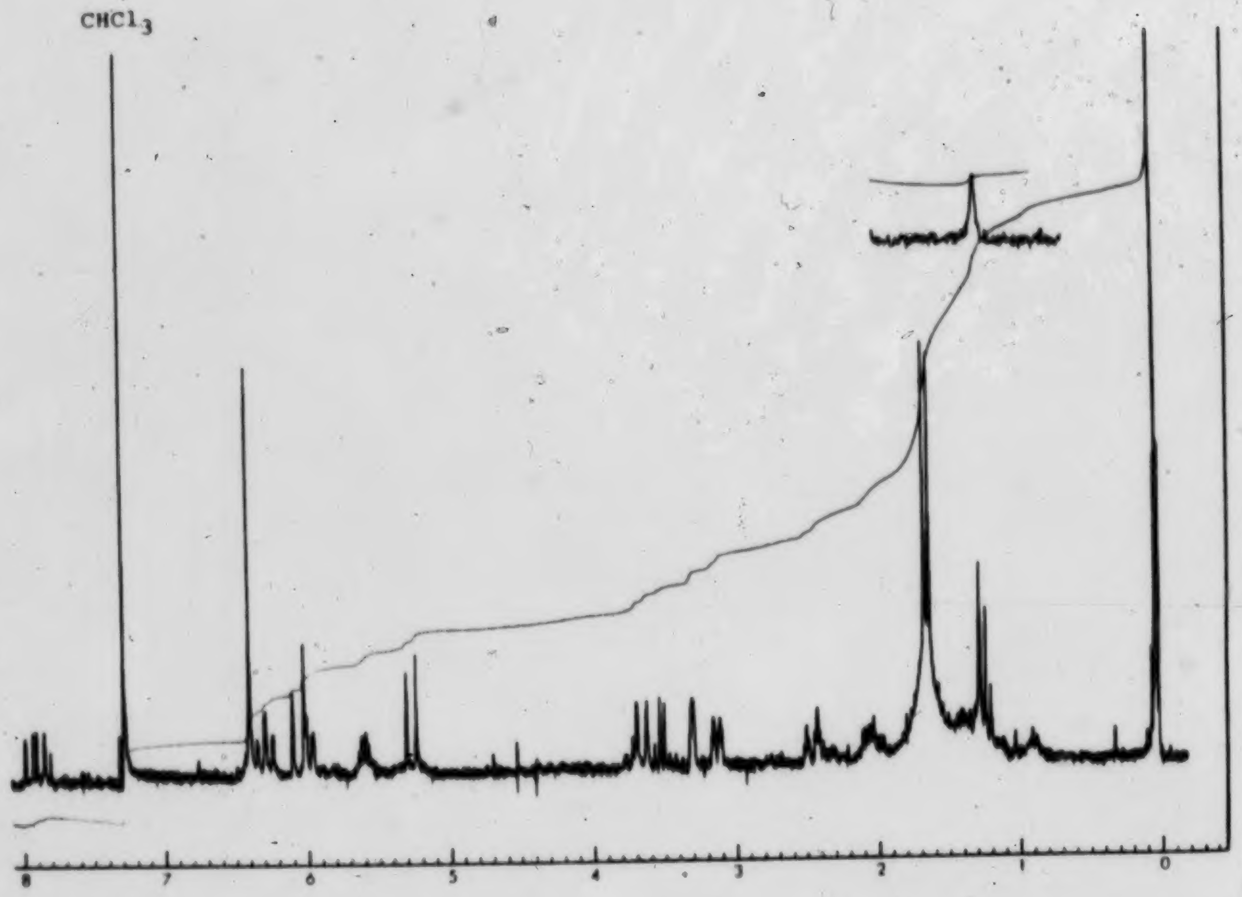


Figure 4. 200 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of monocillin I (**2**)

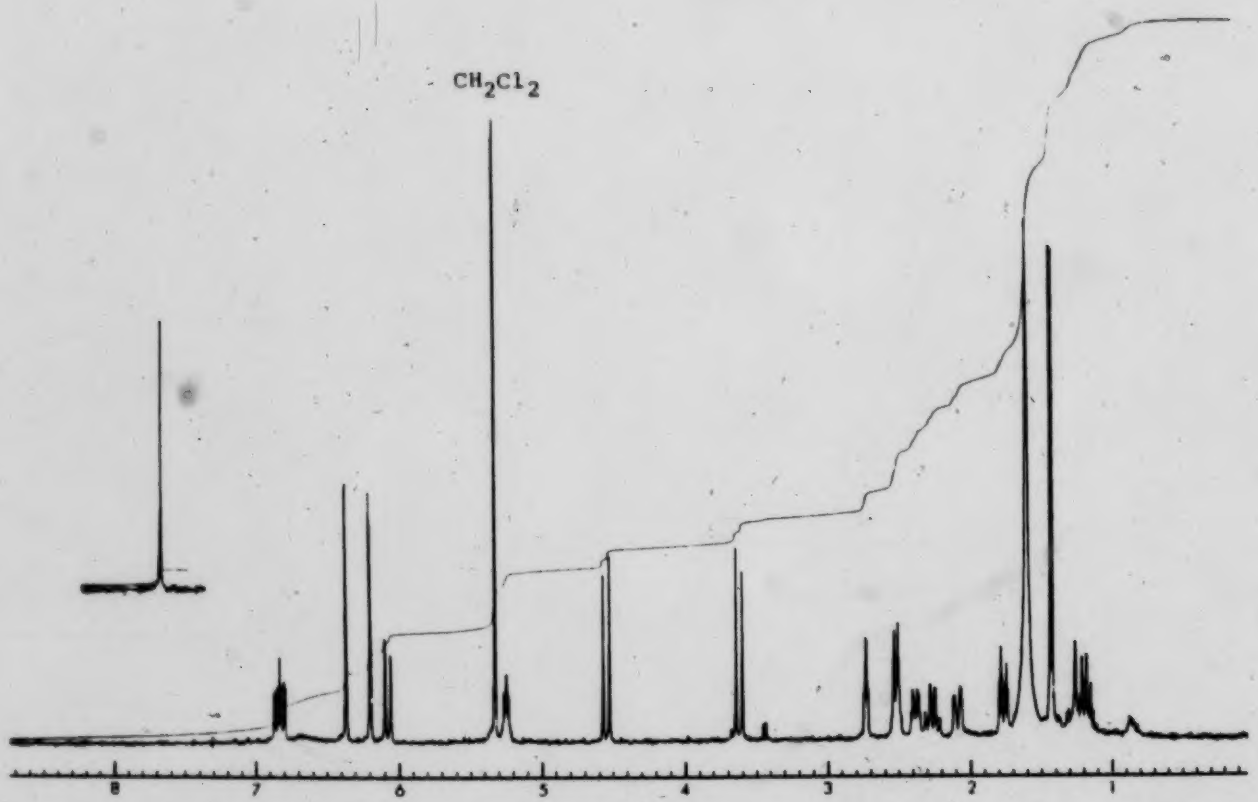
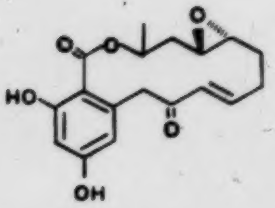


Figure 5. 400 MHz ^1H NMR spectrum ($C\underline{D}_2Cl_2$) of monocillin III (4).

confirmed their identity. The substances proved to be identical by TLC, HRMS and their IR and ^{1}H NMR spectra were superimposable.



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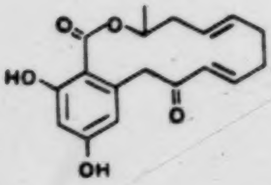
Compound **H** was purified by repeated "dry flash" column chromatography, and obtained as a white solid, mp 197-200°. On the basis of its spectroscopic data and by comparison with that of an authentic sample, **H** was identified as monocillin II (**3**).



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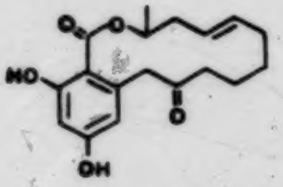
Compound **I** was obtained pure by preparative TLC and identified as monocillin IV (**5**).



5

The IR spectrum of **5** shows a characteristic absorption for a saturated ketone at 1710 cm^{-1},[14,15] and its ^{1}H NMR (Figure 6) displays two olefinic protons as a multiplet at δ 5.47. Comparison of the data for **I** and **5** showed that they were identical by TLC and HRMS and that their IR and ^{1}H NMR spectra were superimposable.

Compound **J** was obtained as a white amorphous solid, mp 130-134°, by preparative TLC purification of a chromatography fraction containing monocillin II (**3**) as the major component. Compound **J** is less polar (R_f 0.59, ethyl ether-Skellysolve B 3:1) than monocillin II (**3**) on TLC.

The HRMS of **J** shows a molecular ion peak at m/z 320 corresponding to a molecular formula of $C_{18}H_{24}O_5$. The base peak at m/z 150 ($C_6H_6O_3$), characteristic of all the monocillins, and corresponding to the fragment ion **13** is also observed.

The IR spectrum of **J** is very similar to that of monocillins IV (**5**) and V (**6**), displaying absorptions typical of a saturated ketone carbonyl (1710 cm^{-1}) and the carbonyl of an α,β-unsaturated and hydrogen-bonded ester (1645 cm^{-1}).[15]

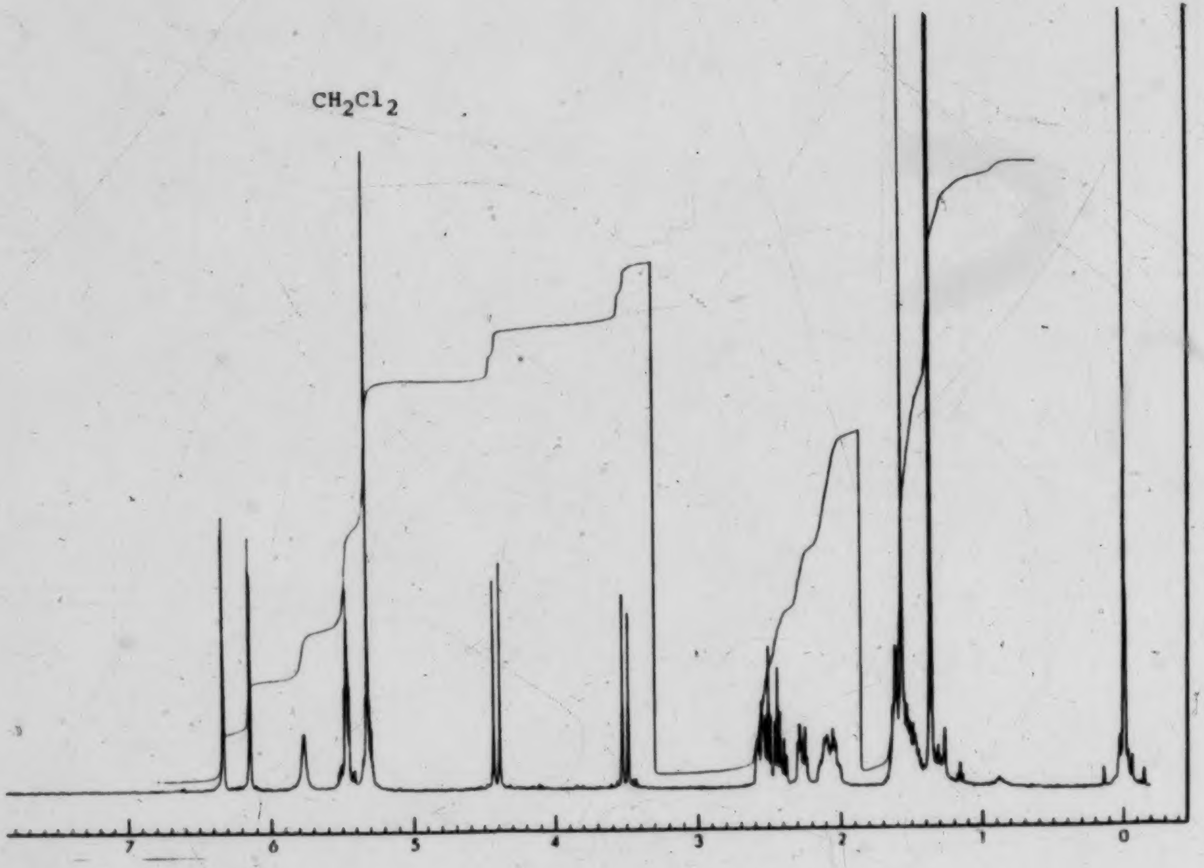
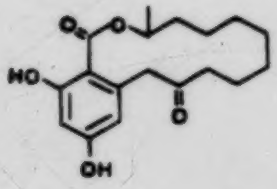


Figure 6. 400 MHz ^{1}H NMR spectrum (CD_2Cl_2) of monocillin IV (5).

In the ^{1}H NMR of compound **J** (Figure 7) there are signals for aromatic protons (δ6.27 and 6.02; doublet each, J = 2.5 Hz), a low field proton (δ5.35, m) characteristic of a hydrogen geminal to oxygen, and benzylic methylene protons (δ4.50 and 3.52). No vinylic protons are observed. The data is consistant with a structure having one less unsaturation than monocillin IV (**5**), thus the structure proposed for compound **J** is that shown below (**14**).



14

Compound **14** has not been isolated previously. We have named this natural product nordinone.

The structure proposed for nordinone (**14**) was confirmed by chemical correlation. Hydrogenation^{6} of monocillin II (**3**, Equation 1) afforded a single product which was identical (TLC, IR, HRMS) with natural nordinone (**14**).

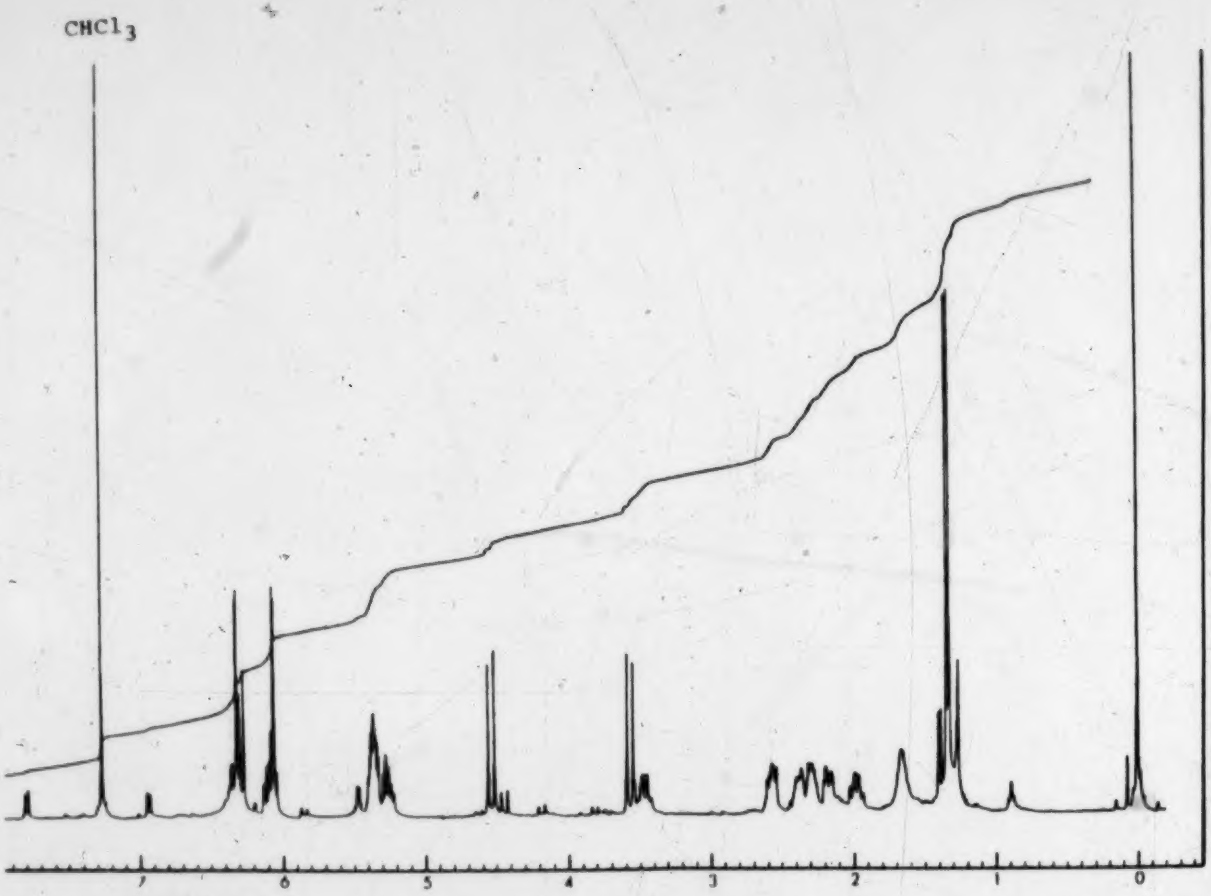
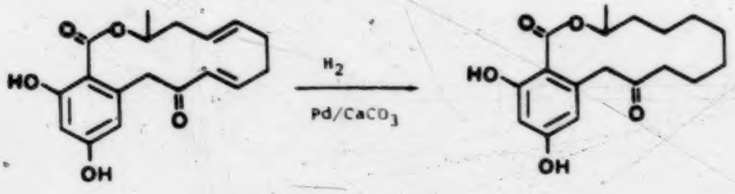


Figure 7. 400 MHz ^{1}H NMR spectrum (C$\underline{D}$Cl$_3$) of nordinone (**14**).



3 **14**

Equation 1

Compound **K** is a polar metabolite (R_f 0.17, ethylether) obtained as large transparent plates, mp 194-196°, after purification of the broth extract of M. nordinii.

The HRMS of **K** has a molecular ion peak at m/z 352 corresponding to the molecular formula $C_{18}H_{24}O_7$. The formula of **K** suggests a monocillin V (**6**) type of structure having an extra molecule of water. The molecular weight of **K** was confirmed as 352 by the presence of a peak at m/z 370 (M^+ + 18) in its CIMS (NH_3). In addition to the molecular ion peak, the HRMS shows fragments at m/z 334 ($C_{18}H_{22}O_6$) and m/z 316 ($C_{18}H_{20}O_5$) corresponding to the loss of one and two molecules of water respectively. The base peak corresponds to the fragment ion at m/z 150 ($C_6H_6O_3$, see **13**).

The IR spectrum of **K** shows absorptions characteristic for "free" (3515 cm^{-1}) and hydrogen-bonded (3400 cm^{-1}) hydroxyl groups,[14,15] ketone carbonyl (1682 cm^{-1}), and the carbonyl of a hydrogen-bonded ortho-hydroxyester (1644 cm^{-1}).[15]

In the ^1H NMR of **K** (Figure 8) the presence of two

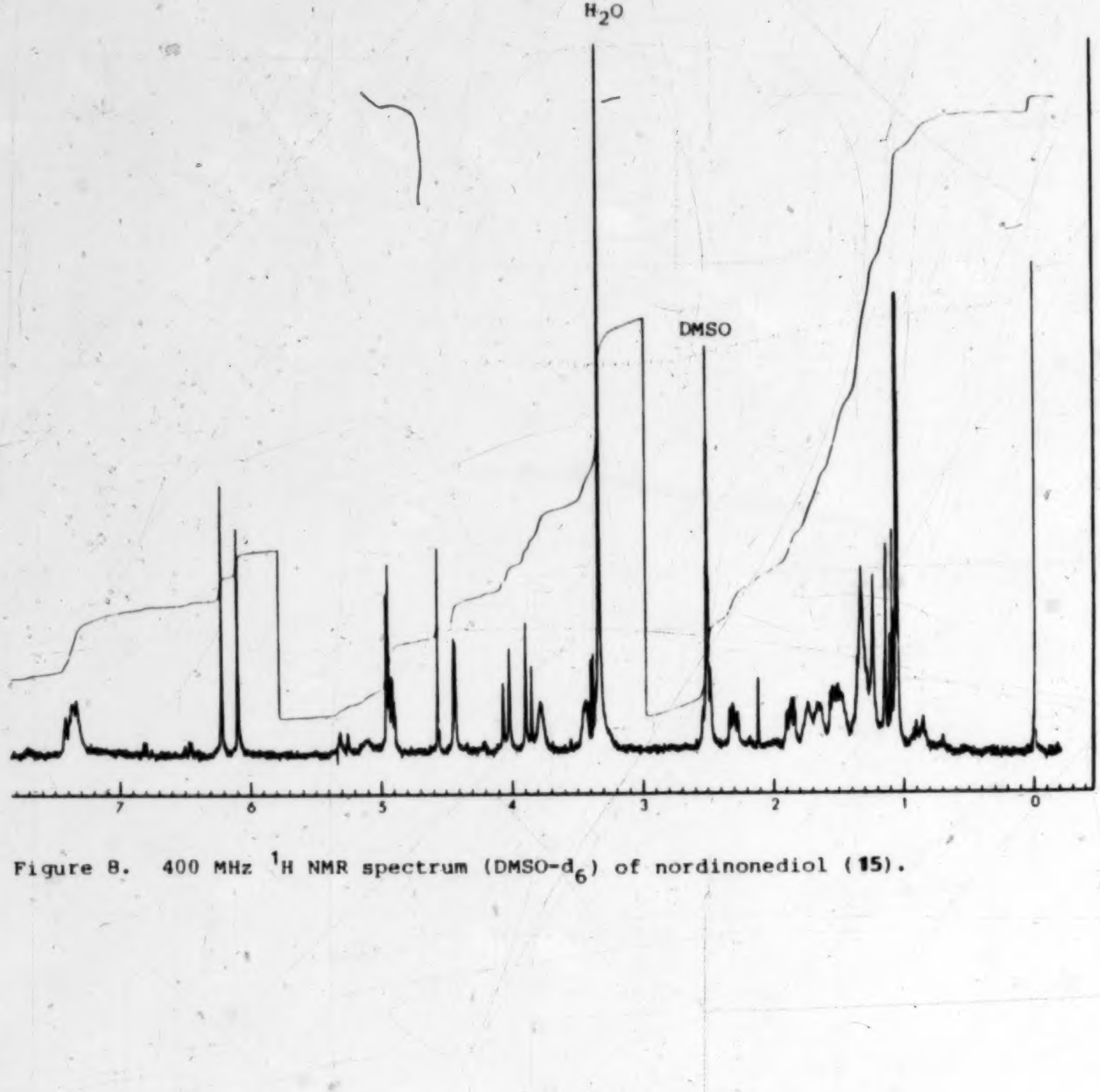
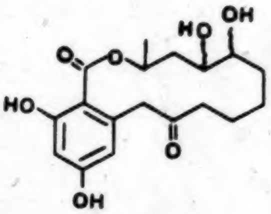


Figure 8. 400 MHz ^{1}H NMR spectrum (DMSO-d_6) of nordinonediol (**15**).

protons geminal to oxygen is detected as one proton signals at δ3.78 (bs) and 3.44 (bs). No signals characteristic of vinylic protons are observed.

Acetylation of compound **K** (acetic anhydride-pyridine) afforded a tetraacetate derivative (**K'**, $C_{26}H_{32}O_{11}$, mol. wt. 520). The IR spectrum of **K'** does not display hydroxyl absorption.

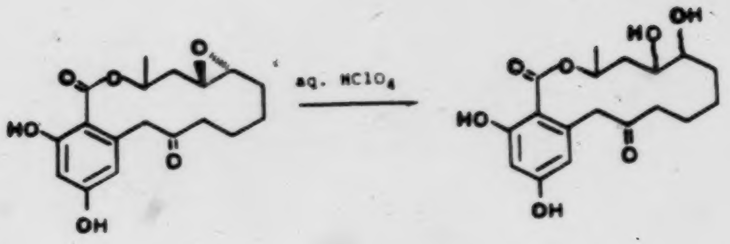
The preceding data suggests that **K** possesses a monocillin V (**6** type structure to which a molecule of water has been added. On this basis structure **15** is proposed for compound **K**.



15

Compound **K** is a new metabolite, which we have named nordinonediol.

Final proof that **K** possesses structure **15** was obtained by treating monocillin V (**6**, obtained by hydrogenation[6] of monocillin I (**2**)) with aqueous acid (Equation 2).



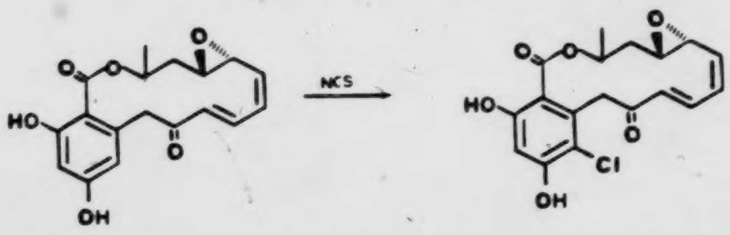
6 **15**

Equation 2

The HRMS, IR, and ^{1}H NMR data of the reaction product is identical with that of the natural product nordinonediol (**15**).

Part II. Chemical Modification of Monocillin I (**2**)

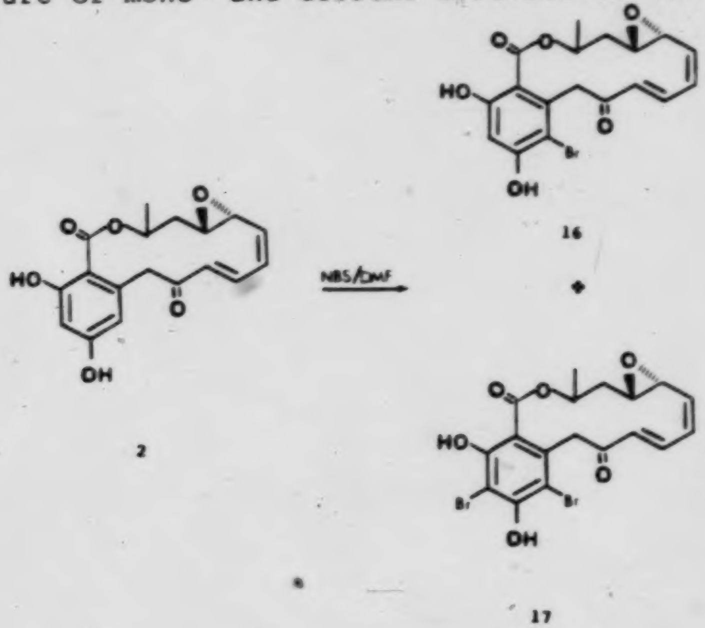
Previous attempts to correlate monocillin I (**2**) with monorden by chlorination of **2** had been unsuccessful.[2] However, chemical correlation was achieved by treatment of monocillin I (**2**) with N-chlorosuccinamide (NCS) in dimethylformamide (DMF, Equation 3). The product of this reaction contained a single major compound by TLC. This compound is identical (TLC behavior and spectroscopic data) with natural monorden (**1**).



2 **1**

Equation 3

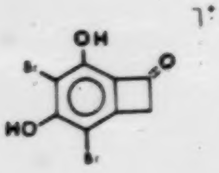
In order to test the antifungal activity of other halogenated monordens, we set about the preparation of the bromo (**16**) and fluoro analogs of monocillin I (**2**). However, treatment of monocillin I (**2**) with N-bromosuccinamide (NBS) in DMF[17] (Equation 4) gave unsatisfactory results. The reaction led to the formation of a mixture of mono- and dibromo derivatives (**16** and **17** respectively). The two components of the mixture were purified by preparative TLC. They were identified on the basis of their HRMS and ^1H NMR spectra.



Equation 4

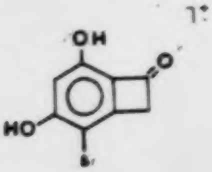
The less polar dibromo derivative **17** showed a molecular ion at m/z 488 ($C_{18}H_{18}O_6Br_2$) and a base peak at m/z 308 ($C_8H_4O_3Br_2$) in its HRMS. This suggests structure **17** and fragment **18** respectively.

The ^{1}H NMR of **17** does not show signals with chemical shifts attributable to aromatic protons.



18

Monobromo derivative **16** shows the expected parent peak at m/z 410 ($C_{18}H_{17}O_6{}^{81}Br$) in its HRMS. The base peak at m/z 229 suggests fragment **19**.



19

The fact that the bromine substituent is located at position 13* is based on the chemical shifts of the C-11* methylene protons (δ 4.80 and 3.95) and the aromatic proton (δ 6.65) in the ^{1}H NMR of **16**. These chemical shift values are very similar to the values observed in the ^{1}H NMR of

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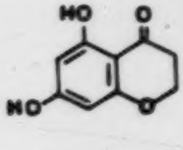
*Numbering according to Mirrington, et.al.(see **1**).[5]

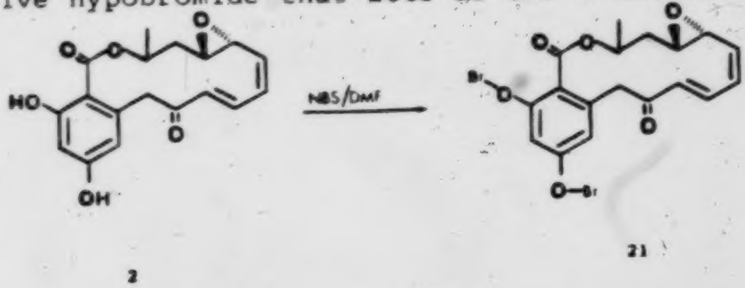
monorden (**1**). It has also been reported[18] that in aromatic substitution reactions of meta-dihydroxy aromatic systems, e.g. type **20**, reaction usually does not occur at



20

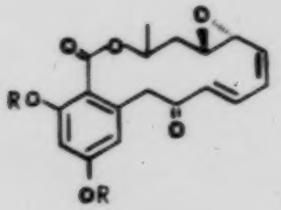
the position ortho to both hydroxyl groups. This lends further support to C-13 as the position of monobromination.

One possible explanation for the formation of the products observed in the NBS bromination of monocillin I (**2**) may be the formation of a hypobromide intermediate (**21**)[19] resulting from the initial reaction of NBS and the phenolic groups (Equation 5). It is then the highly reactive hypobromide that acts as the brominating reagent.

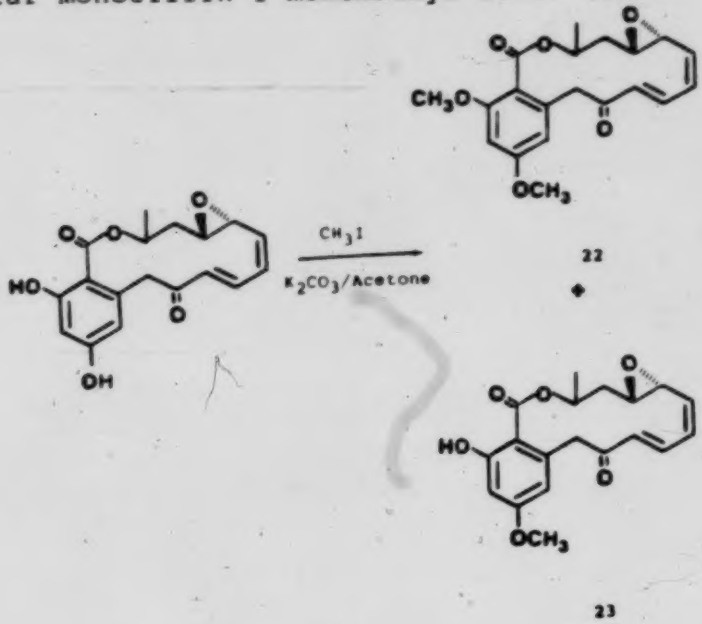


Equation 5

In order to avoid or reduce the formation of the hypobromide and to lower the reactivity of the aromatic ring the phenolic positions were protected. Treatment of

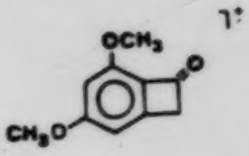


monocillin I (**2**) with methyliodide and potassium carbonate[2] afforded two products (Equation 6). These two compounds, after separation by preparative TLC, were identified as monocillin I dimethyl ether (**22**) and the less polar monocillin I monomethyl ether (**23**).



Equation 6

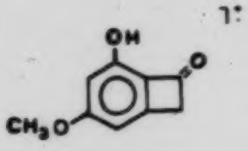
Monocillin I dimethyl ether (**22**) has a molecular formula of $C_{20}H_{22}O_6$ (mol. Wt. 358) as determined from its HRMS. The base peak at m/z 178 ($C_{10}H_{10}O_3$) is consistent with a fragment having structure 24.



24

The IR spectrum of 22 shows no absorption for hydroxyl functionalities, and the carbonyl band, which is no longer hydrogen-bonded, appears at 1720 cm^{-1}.

In the HRMS of the monomethylderivative 23, the parent and base peaks are observed at m/z 344 ($C_{19}H_{20}O_6$) and m/z 164 ($C_9H_8O_3$) corresponding to structures 23 and 25 respectively.

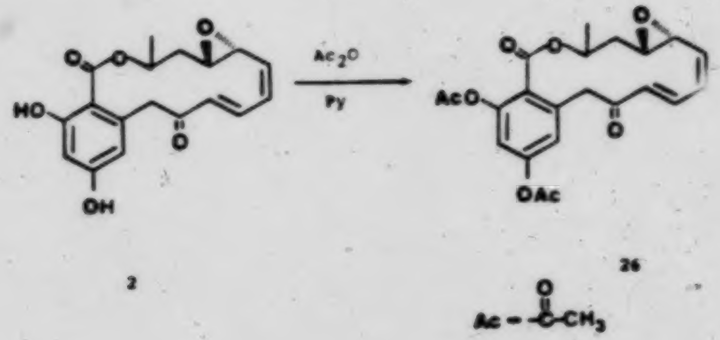


25

The fact that methylation has occurred at position 14 is confirmed by the presence of the α,β-unsaturated hydrogen-bonded carbonyl absorption band (1650 cm^{-1}) in the IR spectrum.

Monocillin I diacetate (26) was prepared by stirring 2 with a mixture of acetic anhydride (Ac_2O) and pyridine

(Py) (Equation 7).



Equation 7

The molecular formula $C_{22}H_{22}O_8$ (414) for **26** was determined from its HRMS, where peaks at m/z 372 and 330, corresponding to the loss of one and two ketene units,[14] are also observed. The characteristic fragment **13** provides the base peak at m/z 150 ($C_6H_6O_3$). Each of the three derivatives (**22**, **23**, and **26**) was subjected to bromination by the NBS/DMF method.[17]

In the case of the dimethyl ether (**22**) and diacetyl (**26**) derivatives only starting material was recovered. The monomethyl ether (**23**), on the other hand, afforded a mixture of the mono- and dibromo derivatives, identified on the basis of their HRMS spectra. At this stage it had become apparent that the lack of water solubility mitigated against the use of the monocillins in the treatment of fungal infections such as Dutch elm disease and these halogenation studies were not pursued.

Part III. Growth of *Monocillium nordinii* Under Different Conditions and HPLC Analysis of the Crude Broth Extracts

Monorden (**1**) and monocillin I (**2**) differ in presence of a chlorine atom in **1**. It has been reported that the chlorine atom in the aromatic ring of monorden (**1**) does not play an important role in the bioactivity of the metabolite since monorden (**1**) has only a slightly stronger antifungal activity compared to that of monocillin I (**2**).[2]

Previous work in these laboratories has shown that the source of chlorine in monorden is the Difco potato dextrose broth (PDB).[2] It was shown by microanalysis to contain 0.3% chloride. It was then reasoned that elimination of chloride from the medium would lead to an increase in the amount of monocillin I (**2**). Addition of other halide ions to the dechlorinated medium might then result in enzymatic halogenation to yield the desired halogenated monocillin derivatives.

The growth of *Monocillium nordinii* under different culture conditions was studied. Each growth was compared on the basis of monocillin I (**2**) and monorden (**1**) production, using the original conditions[1] (PDB containing 0.2% yeast extract) as the control. The seven different media investigated are listed below.

1. PDB containing 0.2% yeast
2. PDB no Cl^-
3. PDB no Cl^- but added Br^-
4. PDB no Cl^- but added F^-
5. Homemade
6. Homemade + Cl^-
7. Homemade + Br^-

The designation "PDB no Cl^-" stands for dechlorinated* medium, while "PDB no Cl^- but added x^-" indicates that a fixed amount* of the corresponding sodium halide was added. "Homemade" medium was prepared in the laboratory using raw potatoes.* "Homemade" medium containing a specified amount of sodium halide is designated "Homemade + x^-".

Crude extracts of Monocillium nordinii contain a number of other compounds in addition to monorden (**1**) and monocillin I (**2**). Thus the use of TLC for qualitative analysis or direct comparison of the various broth extracts is not reliable. A more suitable analysis of the crude extracts can be made using HPLC. The column used was a C-18 reverse phase column, and the carrier eluent was a mixture of acetonitrile-water 35:65. Under these conditions monorden (**1**) and monocillin I (**2**) have retention times of 7.7 and 9.9 minutes (Figures 9 and 10) respectively.

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*For details see Experimental.

Each of the crude extracts was injected after pretreatment.* In a standard culture of Monocillium nordinii (PDB containing 0.2% yeast) twice as much monorden (**1**) as monocillin I (**2**) is produced (Figure 11).

In the "dechlorinated" medium (PDB no Cl^-) the ratio monorden (**1**)-monocillin I (**2**) is 1:1 (Figure 12). Substitution of bromide for chloride ions (PDB, no Cl^- but added Br^-) results in the production of three compounds in a 1:1:1 ratio (Figure 13), the third compound having a retention time intermediate between that of monorden (**1**) and monocillin I (**2**). Purification of the crude extract did not yield bromo-containing metabolites. Addition of fluoride ions (PDB, no Cl^- but added F^-) results in a sharp increase in the production of monorden (**1**), with almost complete inhibition of the production of other metabolites (Figure 14). Use of the "Homemade" medium yields an almost negligible amount of the desired metabolites (Figure 15). However, addition of sodium chloride causes the production of monorden (**1**) and monocillin I (**2**, Figure 16), although in lesser amounts than with commercial PDB. The crude extract from the growth in "Homemade + Br^-" does not show significant

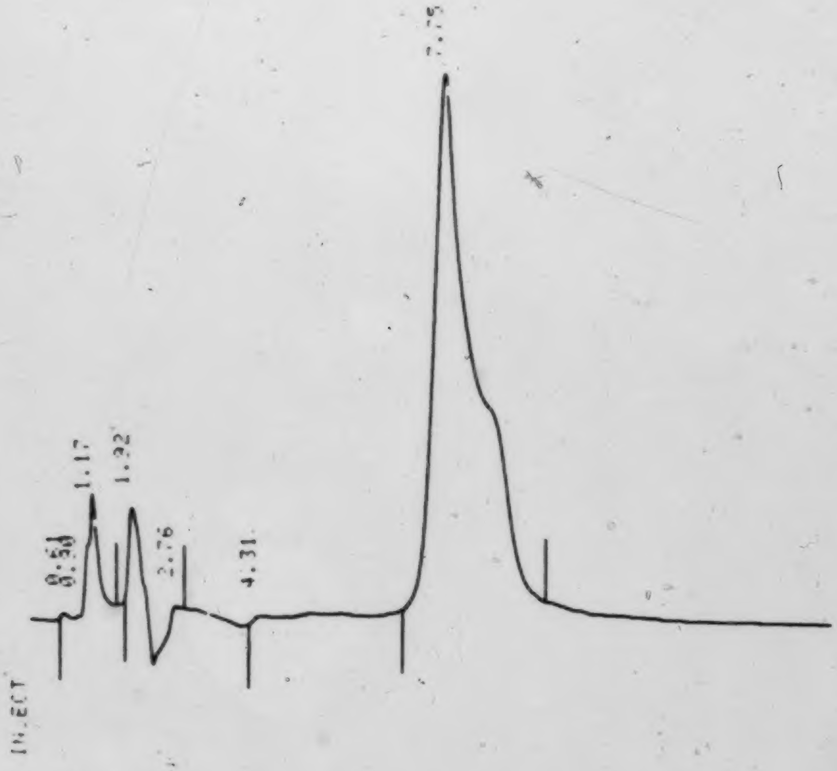

Figure 9. HPLC Chromatogram of monorden (1).

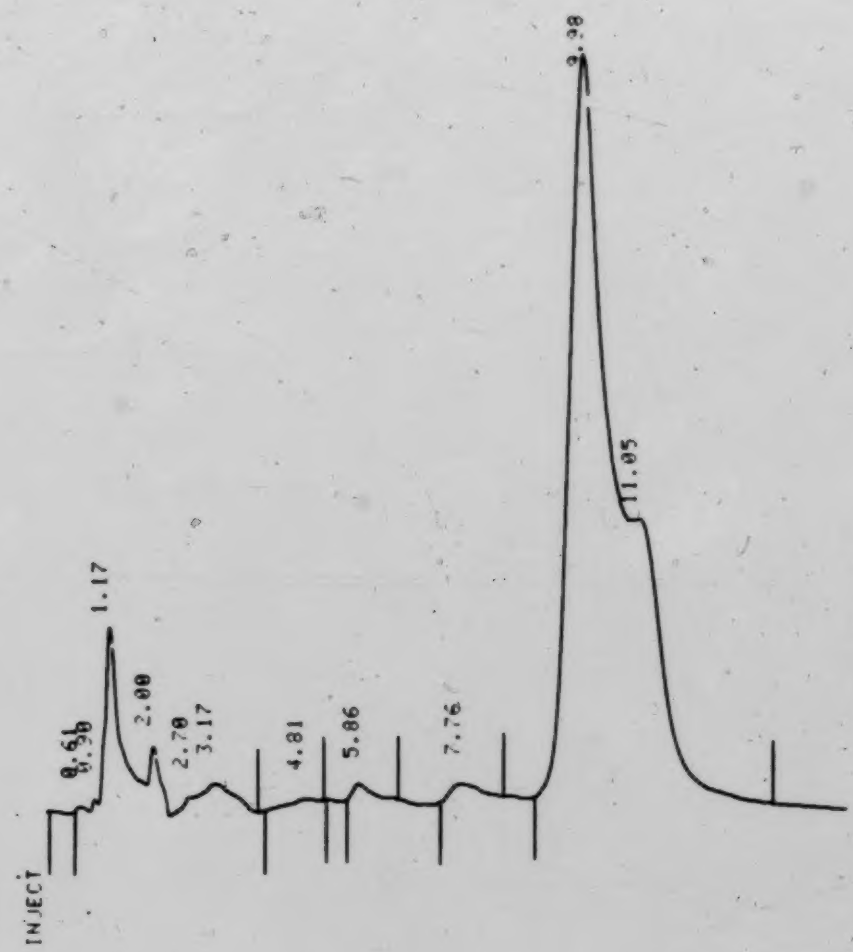

Figure 10. HPLC chromatogram of monocillin I (**2**).

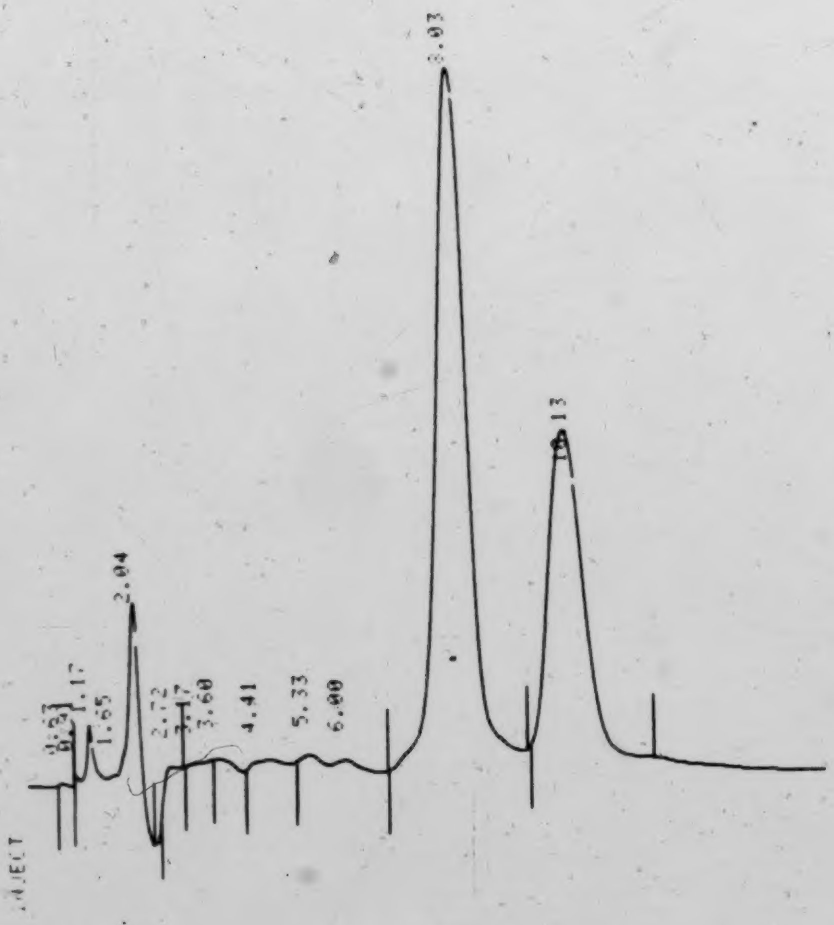

Figure 11. HPLC chromatogram of the PDB containing 0.2% yeast crude.

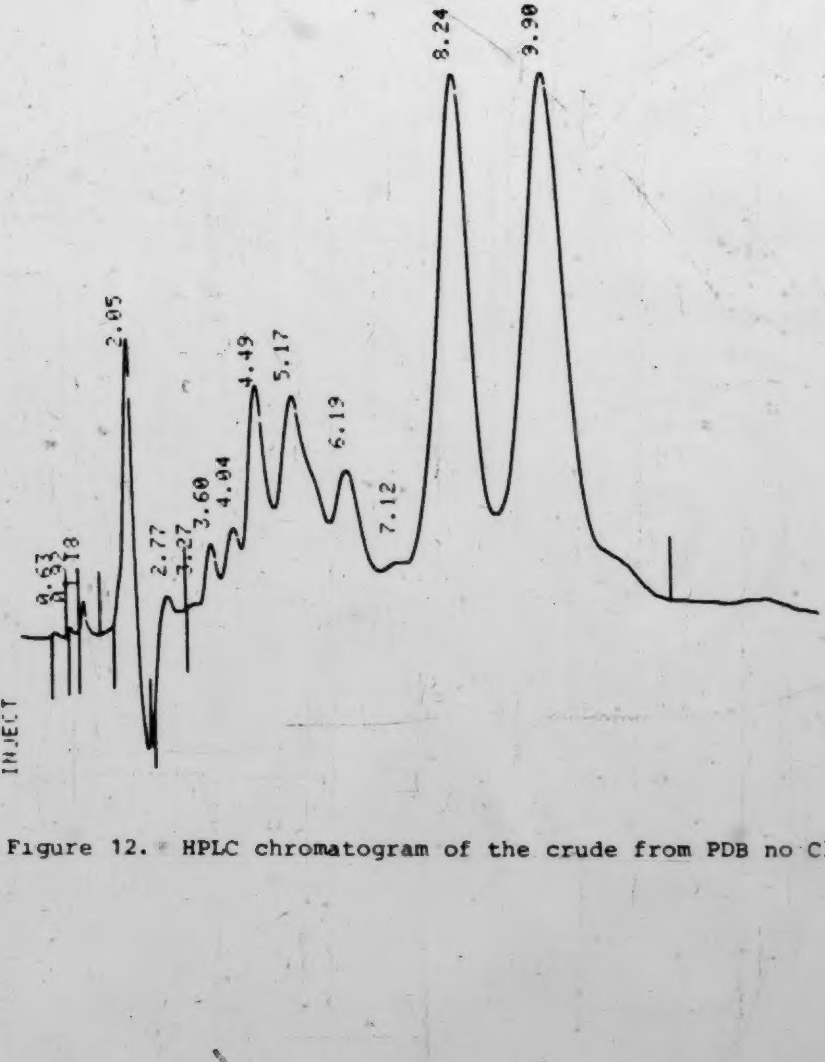

Figure 12. HPLC chromatogram of the crude from PDB no Cl$^-$.

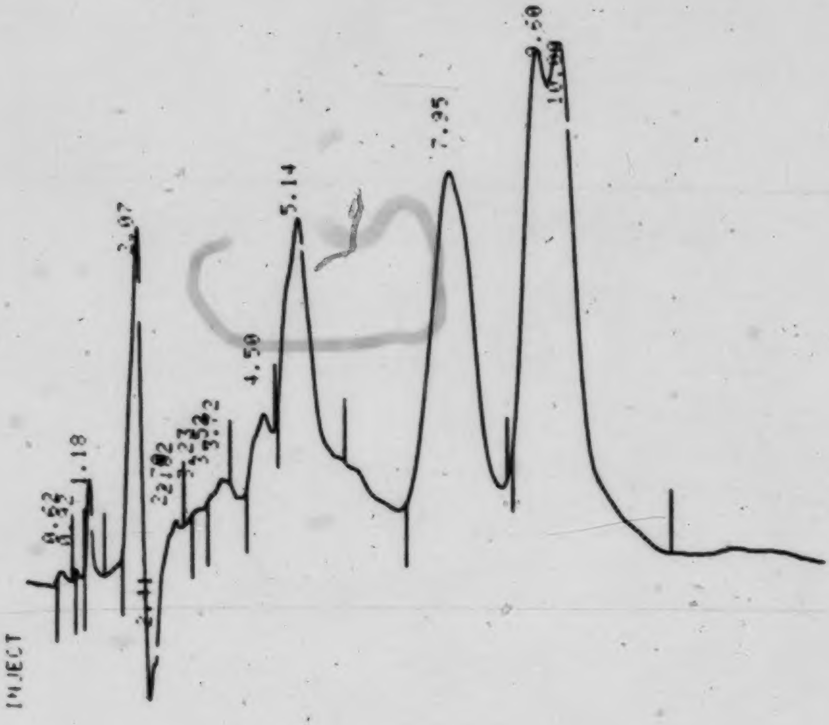

Figure 13. HPLC chromatogram of the crude from PDB, no Cl^-, but added Br^-.

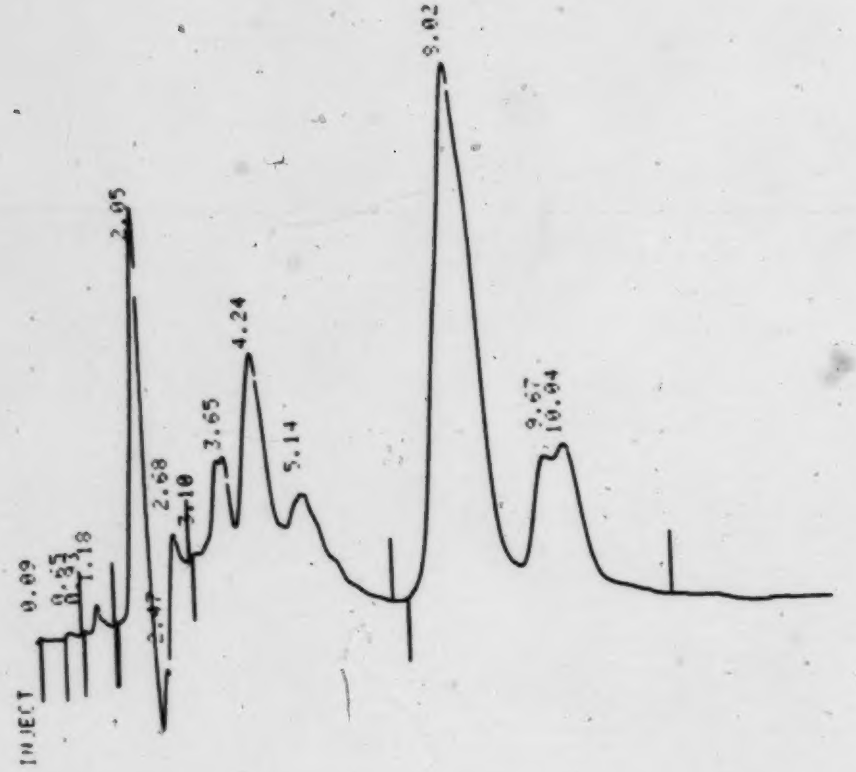

Figure 14. HPLC chromatogram of the crude from PDB, no Cl^-, but added F^-.

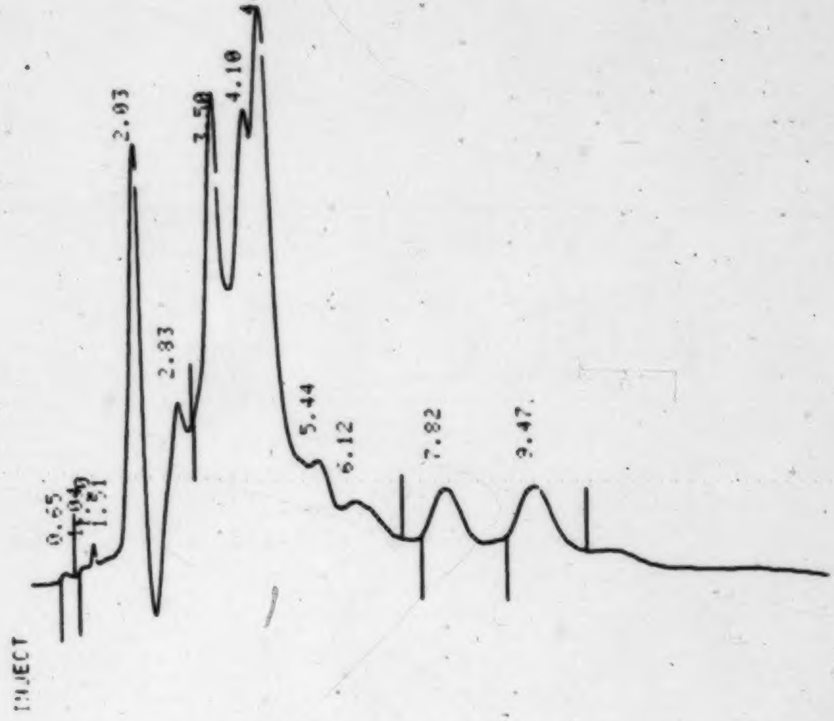

Figure 15. HPLC chromatogram of the crude from "Homemade".

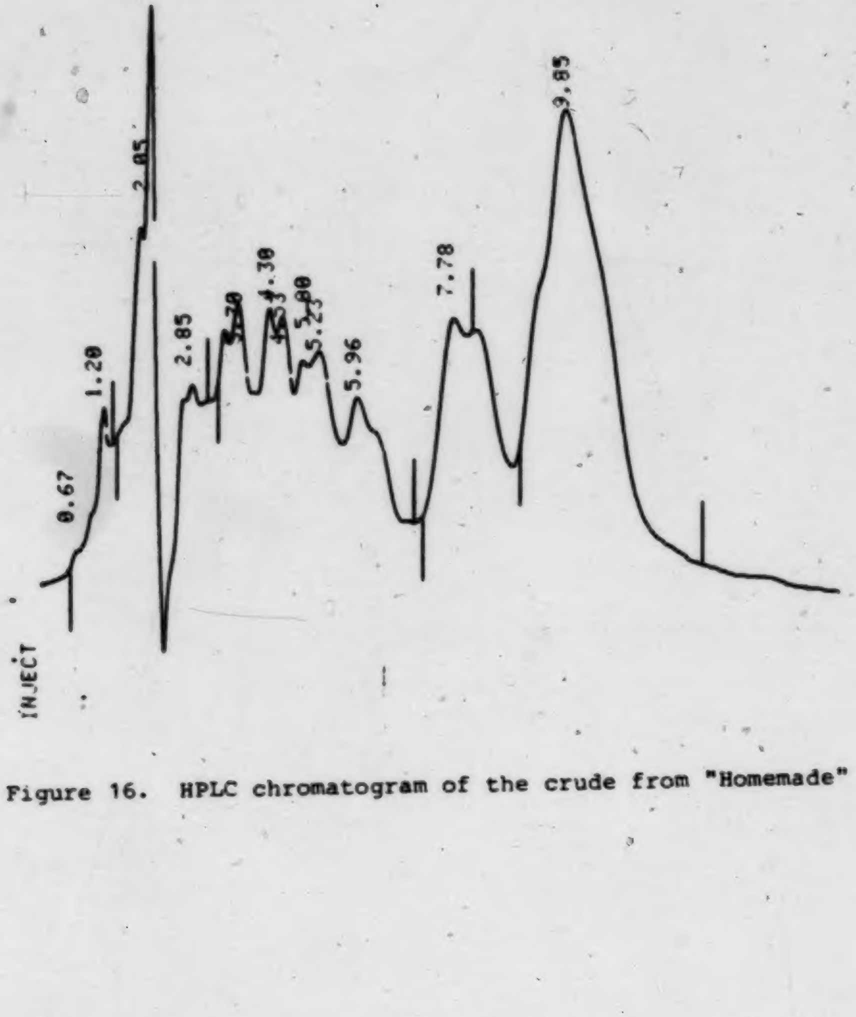

Figure 16. HPLC chromatogram of the crude from "Homemade" + Cl^-.

quantities of any of these metabolites.

Several interesting observations were forthcoming from this work. We did not feel competent to pursue it further, especially since our interest in obtaining the halogenated compounds had declined.

EXPERIMENTAL

Water for fungal culturing was distilled in an all glass apparatus prior to use. Solvents for extraction and purification were distilled prior to use. Skellysolve B refers to Skelly Oil Company light petroleum, bp 62°-70°. E. Merck silica gel 60 (70-230 mesh) was used for column chromatography. Fractions were collected with an ISCO model 200 PUP fraction collector. For flash and "dry flash" column chromatography E. Merck silica gel 60 (230-400 mesh) was employed.

Analytical thin layer chromatography (TLC) was carried out and glass plates (75 × 25 mm or 75 × 50 mm) coated (approximately 0.3 mm) with silica gel G (Terochem, Edmonton) containing 1% electronic phosphor (General Electric, Cleveland) or E. Merck precoated TLC plates of silica gel 60 F-254 (0.25 mm thickness) or E. Merck DC-Alufolien, kieselgel 60 F-254 (0.2 mm thickness). The chromatograms were examined under ultraviolet light (254 or 350 nm) or by placing them in a chamber containing iodine vapour. Visualization was completed by spraying with 30% sulfuric acid followed by careful charring with a heat gun.

Preparative thin layer chromatography was carried out on glass plates (20 × 20 cm, 10 × 20 cm, 5 × 7.5 cm) coated (0.35 mm thickness) with silica gel G (Terochem, Edmonton) containing 1% electronic phosphor (General Electric, Cleveland) or E. Merck precoated TLC plates of silica gel 60 F-254 (20 × 20 cm, 0.25 mm thickness). Materials were detected by visualization under an ultraviolet lamp (254 or 350 nm).

Melting points were determined on a Leitz Weztlar microscope heating stage equipped with Beevy (light intensity control) and Rewiz (temperature control) transformators, and are uncorrected.

Infrared (IR) spectra were recorded on a Nicolet 7199 FT interferometer.

High resolution electron impact mass spectra (HRMS) were recrded on an A.E.I. MS-50 mass spectrometer coupled to a DS-50 data system. Chemical ionization mass spectra (CIMS) and low resolution electron impact mass spectra (LRMS) were obtained using an A.E.I. MS-12 mass spectrometer. Data are reported as m/z (relative intensity). Unless diagnostically significant, peaks with intensities less than 20% of the base peak are omitted.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured on a Varian HA-100 spectrometer with a Digilab FTS/NMR-3 data system. High field ^{1}H NMR and ^{13}C

NMR spectra were recorded on Bruker WH-200 or WH-400 spectrometers with an Aspect 2000 computer system. All nuclear magnetic resonance measurements employed tetramethylsilane (TMS) as an internal standard.

Centrifugation of the broth was carried out using a Dupont Instruments Sorvall RC-5B refrigerated superspeed centrifuge, equipped with a GSA rotor.

Growth of *Monocillin nordinii* Cultures and Extraction of Metabolites

Stock cultures of *Monocillin nordinii* (C-690) were obtained from the Northern Forestry Research Center, Edmonton, and maintained in slant tubes of Difco potato dextrose agar* (PDA) at 5°C. *M. nordinii* was asceptically transferred from the stock cultures to plates of PDA. After two weeks growth at room temperature the cultures were mature. Sterilized water (2 mL) was added and the surface of the plate scraped with a sterile needle to make a mycelium suspension. This suspension was used to inoculate 1 liter of Difco potato detroxe broth* (PDB) containing 0.2% of yeast extract contained in a two liter Fernbach flask. After inoculation, the liquid still cultures were kept at room temperature for 14 days. The culture broth was decanted from the mycelium and concentrated to 1/20 of its original volume under reduced pressure. The concentrated broth was extracted with ethyl ether for 24 h, and the ether solution dried (anh. sodium sulfate) and concentrated to give crude broth extract (0.3270 g per liter).

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*For composition see Appendix of Experimental.

The mycelium was dried in the fumehood and extracted with chloroform for 24 h in a Soxhlet extractor. Elimination of the solvent afforded crude mycelial extract (0.2000 g per liter).

Preparation of Dechlorinated PDB[20]

The necessary amount of silver nitrate (0.3493 g) to precipitate all the chloride present (assuming 0.5% was present) was dissolved in distilled water (10 mL) and added to a solution of PDB (60 mL). The solution was heated to coagulate the precipitate and a gray-blueish suspension was obtained. This suspension was allowed to cool, then autoclaved (15 min, 121°, 15 psi). Centrifugation (5000 pm, 30 min, 20°C) of the suspension gave a black precipitate with a dark pink supernatant. The supernatant was decanted, diluted to 1 liter and divided into five 200 mL portions. To each portion the corresponding salts were added, the solutions were sterilized and the cultures inoculated with the fungus as previously described.

Work up of the cultures and extraction of the metabolites were performed in the usual manner.

Preparation of "Homemade" PDB[21]

In a two liter beaker 300 g of finely diced potatoes were boiled in 500 mL of distilled water until thoroughly cooked. The solids were removed by filtration through cheesecloth and water was added to the filtrate to make 1 liter. Prior to sterilization 20 g of glucose were added.

Purification of the Metabolites

A wide range of column chromatography variations were surveyed when attempting the purification of the metabolites from the broth and mycelium extracts. A representative description of them, together with their advantages or disadvantages, is listed below.

For the purification of either the broth or mycelium crude extracts, a column was packed with a suspension of silica gel (adsorbent-sample ratio = 200:1) in chloroform. The crude extract was dissolved in the minimum amount of chloroform-methanol and applied at the top of the column. Separation was initiated using chloroform as the eluent and continued by gradually increasing the polarity of the solvent by the addition of methanol (0.5, 1.0, 2.5, 5.0, 10%, approximately 1 liter each time). The flow rate was controlled to 15 mL/h and 5 mL fractions were collected using an automatic fraction collector. The fractions were monitored by TLC and the similar ones combined. In this manner 10-15 fractions were usually obtained, most of them complex mixtures which had to be further purified. The main problem with this separation was given by the presence of yellow pigments contaminating almost every fraction. These were difficult to eliminate even by repeated recrystallization of the metabolites.

Since the pigments seemed to be non-polar, a preliminary "reverse phase" purification of the crude was attempted. The crude was dissolved in the minimum amount of methanol and applied at the top of a column packed with a suspension of Sephadex LH-20 (prepacked, 1 m long, 3.5 cm diameter) in the same solvent. Methanol was used as the eluent. The flow rate was controlled to 15 mL/h and 5 mL fractions were collected using an automatic fraction collector. The fractions were monitored by TLC, similar fractions being combined to give 6-10 major fractions. This procedure did not lead to increased purity of fractions or decreased presence of the pigments. Further purification of the fractions yielded some pure metabolites.

In view of these results a new technique was developed and used in the purification of the crude extracts. This technique has been labelled "dry flash" column chromatography* and a typical separation can be described as follows: 212 mg of the crude extract were adsorbed on silica gel and placed on top of an 8 in long, 3 cm diameter, silica gel column. Final packing, air expulsion and flow rate control (2 in/min) was performed by passing Skellysolve B through the column (3×). Separation of the mixture was carried out using a 3:1 mixture of ethyl ether-Skellysolve B. Fractions of 20 mL were collected manually, monitored by TLC and, if similar, combined giving 10-15 final fractions. Usually monorden (**1**) and monocillin III (**4**) were obtained in pure, crystalline form. The other monocillins and metabolites were obtained readily by further fraction purification. The problem of the pigment presence was completely eliminated by the use of this technique.

Preparation of HPLC Samples

A small amount of activated charcoal was added to a 1% solution (in chloroform) of the sample to be analyzed. The mixture was boiled for five minutes and then allowed to cool. The suspension was filtrated through a silica gel (70-270 mesh) bed and the solvent evaporated under reduced pressure. The recovery being approximately 48%.

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*For a detailed description see Isolation and Identification of Metabolites.

The sample was then redissolved in a mixture of acetonitrile-methanol 3:1 in a concentration of 1 mg/mL. For the HPLC analysis 100 μL of sample were injected, the eluent was a mixture of water-acetonitrile 65:35 with a flow rate of 2 mL/min, working with the UV detector set at 240 nm and using a run time of 15 min.

Isolation and Identification of Metabolites

Dry flash column chromatography. This method was developed in this laboratry to separate crude mixtures or complex fractions which are not soluble in the eluting solvent. The procedure is a combination of two techniques: dry column and flash chromatography. The chromatography column is packed as described by Still[22] omitting the sand at the top of the column. The sample adsorbed on silica gel (ratio sample-adsorbent 1:7) is loaded in a thin, even band, and then covered with approximately 2 1/2 in of sea sand. A non polar solvent is passed through until all of the air is expelled from the column, and the solvent flow rate is adjusted. Separation proceeds as described by Still[22] using the chosen solvent system as eluent. We have found that mixtures of Skellysolve B-acetone, ethyl ether-Skellysolve B, and benzene-acetone give excellent results for separation of *A. brassicae* and *M. nordinii* extracts. Compounds usually crystallize when the fractions are concentrated. The column length employed varied for complex separations (8 in) or for partial fractionations (4 in). This method was employed to effect separations of crude extracts (350 mg) or purification of reaction products (2 mg).

Isolation of Sterigmatocystin (**8**)

Purification of the mycelium extract by silica gel column chromatography (chloroform-methanol, gradient elution 0.5-10%) yielded six major fractions. The fourth fraction (241.0 mg) showed (TLC) the presence of a non-polar yellow pigment. This fraction was further purified by silica gel column chromatography (ethyl ether-toluene, gradient elution 1-50%) from where a second set of six fractions was obtained. Sterigmatocystin (**8**, 2 mg) was obtained pure by preparative TLC (20×20 cm, toluene-acetone-acetic acid 75:25:1) of fraction **E** (13.6 mg). TLC: R_f 0.86 (chloroform-ether 1:1). IR ($CHCl_3$ cast): 3200, 1645, 1625, 1610, 1585, 1460 cm^{-1}. ^{1}H NMR (100 MHz, $C\underline{D}Cl_3$): δ 3.98 (3H, s), 4.80 (1H, dt, 2, 8 Hz), 5.44 (1H, t, 2.5 Hz), 6.43 (1H, s), 6.50 (1H, dd, 2, 1.5 Hz), 6.72 (1H, dd, 1, 8 Hz), 6.82 (1H, dd, 1, 8 Hz), 6.82 (1H, d, 8 Hz), 7.50 (1H, dd, 8, 7.5 Hz), 13.25 (1H, s).

Isolation of Ergosterol endoperoxide (**9**)

Fraction 2 (92.4 mg) from the purification of the mycelium extract showed a major component. Dry flash column chromatography (ethyl ether-Skellysolve B 3:1, 6 in long, 2 cm diameter) purification yielded Ergosterol endoperoxide (**9**, 5 mg) as white crystals, mp 162-163°. TLC: R_f 0.48 (ethyl ether-Skellysolve B 3:1, 2×). IR ($CHCl_3$ cast): 3420, 2955, 2871, 1457, 1374, 1100, 1040 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_2/C\underline{D}_3O\underline{D}$): δ6.48 (1H, dd, 6, 6 Hz), 6.22 (1H, dd, 6, 6 Hz), 5.22 (2H, m), 3.84 (1H, bs). HRMS: m/z calcd for $C_{28}H_{44}O_3$ (M^+): 428.3290; found: 428.3288(1), 410(1), 396(30), 378(1), 271(3), 253(5), 211(3).

Isolation of succinic acid (**10**)

Purification of the broth extract by reverse phase column chromatography using a Sephadex LH-20 column (prepacked, 1 m long, 3.5 cm diameter) and methanol as the eluting solvent afforded five major fractions. Succinic acid (**10**, 12 mg) crystallized (ethyl ether) from fraction 2 (276 mg) as crystalline prisms, mp 185-187° (lit. 187-189°). IR ($CHCl_3$ cast): 3000, 1691 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ2.40 (4H, s), 10.92 (2H, s). HRMS: m/z calcd. for $C_4H_6O_4$ (M^+): 118.0266; found: 118.0260(1), 100(71), 74(71), 55(100).

Isolation of uracil (**11**)

Uracil (**11**, 8 mg) crystallized as a beige amorphous powder, mp > 350° (lit. > 300°), from fraction 3 of the reverse phase column chromatography purification of the broth extract. IR (KBr disc): 3100, 1716, 1730, 830 cm^{-1}. ^{1}H NMR (100 MHz, DMSO-d_6): δ5.45 (1H, d, 8 Hz), 7.40 (1H, d, 8 Hz), 11.06 (2H, bs). HRMS: m/z calcd. for $C_4H_4N_2O_2$ (M$^+$): 112.0273; found: 112.0270(100), 69(21).

Isolation of monorden (**1**)

Purification of the crude broth extract by dry flash column chromatography (ethyl ether-Skellysolve B 3:1) afforded 18 fractions. Fractions containing **1** (as determined by TLC) were combined and allowed to stand in the fumehood. Monorden (**1**, 15 mg) crystallized as colorless needles, mp 175-178° (lit. 195°). TLC: R_f 0.69 (dichloromethane-methanol 98:2, 2×), 0.40 (ethyl ether-Skellysolve B 3:1, 2×). IR ($CHCl_3$ cast): 3300, 1655, 1630, 1603, 1304, 1240, 1105 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$): δ11.12 (1H, s), 7.49 (1H, dd, 10, 16 Hz), 6.65 (1H, s), 6.20 (1H, s), 6.19 (1H, t, 11 Hz), 6.11 (1H, d, 15.5 Hz), 5.83 (1H, dd, 3, 11 Hz), 5.51 (1H, m), 4.81 (1H, bd, 16 Hz), 3.92 (1H, bs), 3.18 (1H, bs), 2.96 (1H, dt, 3, 9 Hz), 2.37 (1H, dt, 3.5, 15.5 H), 1.96 (1H, m), 1.54 (3H, d, 7 Hz). HRMS: m/z calcd. for $C_{18}H_{17}O_6{}^{35}Cl$ and $C_{18}H_{17}O_6{}^{37}Cl$ (M$^+$): 364.0713 and 366.0684; found: 364.0709(37), 366.0687(12), 184(100), 186(34).

Isolation of monocillin I (**2**)

Separation of the crude broth and mycelial extracts using dry flash column chromatography (ethyl ether-Skellysolve B 3:1) was followed either by preparative TLC (ethyl ether-Skellysolve B 3:1, 2×) or a second dry flash column (ethyl ether-Skellysolve B 3:2) to give monocillin I (**2**, 17 mg) as an amorphous white powder, mp 75-77°. TLC: R_f 0.43 (ethyl ether-Skellysolve B 3:1), 0.55 (ethyl ether-Skellysolve B 3:1, 2×), 0.41 (Skellysolve B-ethyl acetate 80:20, 4×). IR ($CHCl_3$ cast): 3340, 1649, 1620, 1590, 1448, 1310, 1260 cm^{-1}. ^1H NMR (100 MHz, $C\underline{D}Cl_3/C\underline{D}_3O\underline{D}$): δ 11.30 (1H, s), 7.87 (1H, dd, 16, 12 Hz), 6.42 (1H, d, 2 Hz), 6.41 (1H, d, 2 Hz), 6.30 (1H, dt, 2, 6 Hz), 6.06 (1H, d, 16 Hz), 5.97 (1H, dd, 2, 5 Hz), 5.60 (1H, m), 5.28 (1H, d, 14 Hz), 3.60 (1H, d, 14 Hz), 3.31 (1H, bs), 3.14 (1H, dt, 3, 10 Hz), 2.67 (1H, dt, 4, 15 Hz), 2.04 (1H, m), 1.67 (3H, d, 6 Hz). HRMS: m/z calcd. for $C_{18}H_{18}O_6$ (M^+): 330.1103; found: 330.1101(21), 150(100).

Isolation of monocillin III (**4**)

Purification by dry flash column chromatography (ethyl ether-Skellysolve B 3:1) of the crude extract yielded 18 fractions. Monocillin III (**4**, 10 mg) crystallized from these chromatography fractions as white crystals, mp 205-207° (lit. 204-205). TLC: R_f 0.56

(Skellysolve B-ethyl acetate 1:1), 0.18 (ethyl ether-Skellysolve B 3:1, 2×). IR ($CHCl_3$ cast): 3230, 1644, 1620, 1313, 1261 cm^{-1}. ^1H NMR (200 MHz, $C\underline{D}Cl_2$): δ11.10 (1H, s), 6.82 (1H, ddd, 4, 11, 16 Hz), 6.36 (1H, d, 2.5 Hz), 6.18 (1H, d, 2.5 Hz), 6.06 (1H, dd, 1.5, 16 Hz), 5.25 (1H, m), 4.52 (1H, d, 17 Hz), 3.60 (1H, d, 17 Hz), 2.72 (1H, m), 2.50 (1H, dt, 3, 10 Hz), 2.38 (1H, m), 2.24 (1H, m), 2.07 (1H, dt, 4, 15 Hz), 1.57 (1H, dt, 4, 16 Hz), 1.42 (3H, d, 6.5 Hz). HRMS: m/z calcd. for $C_{18}H_{20}O_6$ (M^+): 332.1260; found: 332.1261(46), 150(100).

Isolation of monocillin II (**3**)

Dry flash column chromatography (ethyl ether-Skellysolve B 3:1) separation of the crude broth extract followed by purification using dry flash column chromatography (ethyl ether-Skellysolve B 3:2) of the fractions containing monocillin II (**3**) gave monocillin II (**3**, 15 mg) as an amorphous white solid, mp 198-200°. TLC: R_f 0.71 (ethyl ether), 0.75 (Skellysolve B-ethyl acetate 1:1), 0.47 (ethyl ether-Skellysolve B 3:1), 0.66 (ethyl ether-Skellysolve B 3:1, 2×). IR ($CHCl_3$ cast): 3400, 1645, 1616 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}_2Cl_2/C\underline{D}_3O\underline{D}$): δ6.64 (1H, m), 6.31 (1H, d, 3 Hz), 6.20 (1H, d, 3 Hz), 5.83 (1H, dd, 1, 15 Hz), 5.25 (2H, m), 4.13 (1H, d, 16 Hz), 3.79 (1H, d, 16 Hz), 3.36 (1H, s), 2.67 (1H, m). HRMS: m/z calcd. for $C_{18}H_{20}O_5$ (M^+): 316.1311; found: 316.1306(25), 150(59).

Isolation of monocillin IV (**5**)

Purification of the broth extract by dry flash column chromatography (ethyl ether-Skellysolve B 3:1) afforded 9 fractions. Further purification of fraction 2 (5.4 mg) by preparative TLC (10×10 cm, ethyl ether-Skellysolve B 3:2) afforded monocillin IV (**5**, 3 mg). TLC: R_f 0.55 (ethyl ether-Skellysolve B 3:1). IR ($CHCl_3$ cast): 3350, 2927, 1710, 1657, 1590, 1450 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}_2Cl_2$): δ 11.60 (1H, s), 6.33 (1H, d, 3 Hz), 6.15 (1H, d, 3 Hz), 5.77 (1H, m), 5.47 (2H, m), 5.30 (1H, m), 4.42 (1H, d, 17 Hz), 3.55 (1H, d, 17 Hz), 2.6-2.0 (6H, m), 1.37 (3H, d, 6 Hz). HRMS: m/z calcd. for $C_{18}H_{22}O_5$ (M^+): 318.1467; found: 318.1466(27), 150(100).

Isolation of nordinone (**14**, 3,4,5,6,7,8,9,10-octahydro-14,16-dihydroxy-3-methyl-1H,2-benzoxacyclotetradecin-1,11(12H)-dione)

The mixture of monocillin II (**3**) and a less polar component (40.2 mg) was separated by preparative TLC (20×20 cm, ethyl ether-Skellysolve B 3:1, 2×). The less polar compound (6 mg), identified as nordinone (**14**), was obtained as a white amorphous powder, mp 130-134°. TLC: R_f 0.59 (ethyl ether-Skellysolve B 3:1). IR ($CHCl_3$ cast): 3360, 1710, 1645, 1619. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ 6.27 (1H, d, 2.5 Hz), 6.02 (1H, d, 2.5 Hz), 5.35 (1H, m), 4.50 (1H, d, 18 Hz), 3.52 (1H, d, 18 Hz), 2.65-

1.6 (10H, m), 1.35 (3H, d, 7 Hz). HRMS: m/z calcd. for $C_{18}H_{24}O_5$ (M^+): 320.1624; found: 320.1614(3), 150(100). CIMS (NH_3): 339(20), 338(83), 322(20), 321(100).

Hydrogenation of monocillin II (**3**)

Impure monocillin II (**3**, 28 mg) was dissolved in ethanol (10 mL) and Palladium over calcium carbonate (10 mg) was added. The reaction mixture was stirred in a hydrogen atmosphere for 50 min. A TLC of the mixture did not show the presence of the starting material. The catalyst was removed by gravity filtration, and the solvent removed *in vacuo* to yield the crude product (21.7 mg). Purification of this product by dry flash column chromatography (ethyl ether-Skellysolve B 65:35, 15 cm long, 0.5 cm diameter) afforded an amorphous white powder, mp 135-138°, identified as nordinone (**14**, 0.8 mg) on the basis of its TLC behavior and spectroscopic data.

Isolation of nordinonediol (**15**, 3,4,5,6,7,8,9,10-octahydro-5,6,14,16-tetrahydroxy-3-methyl-1H,2-benzoxacyclotetradecin-1,11(12H)-dione)

Reverse phase column chromatography purification of the broth extract using Sephadex LH-20 as the adsorbent and methanol as the eluent afforded 5 major fractions. Further purification of fraction 2 (276 mg) by flash chromatography (chloroform-methanol, gradient elution 0.5-

3%) yielded 8 fractions. Nordinonediol (**15**, 7 mg) crystallized from fraction 7 (46 mg) as large colorless crystals, mp 194-196°. TLC: R_f 0.17 (ethyl ether). IR (KBr disc): 3515, 3400, 3182, 2965, 2935, 1682, 1644, 1469, 1264 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ10.38 (1H, s), 9.92 (1H, s), 6.20 (1H, d, 2 Hz), 6.08 (1H, d, 2 Hz), 4.9 (1H, d, 2.5 Hz), 4.90 (1H, m), 4.42 (1H, d, 2.5 Hz), 4.04 (1H, d, 18 Hz), 3.86 (1H, d, 18 Hz), 3.78 (1H, bs), 3.44 (1H, bs), 2.3-1.4 (6H, m), 1.04 (3H, d, 6 Hz). HRMS: m/z calcd. for $C_{18}H_{24}O_7$ (M^+): 352.1522; found: 352.1524(13), 334(4), 316(4), 150(100). CIMS (NH_3): 370(100), 353(30), 335(26).

O,O,O,O-Tetraacetylnordinonediol (**K'**, 3,4,5,6,7,8,9,10-octahydro-5,6,14,16-tetraacetoxy-3-methyl-1H,2-benzoxacyclotetradecin-1,11(12H)-dione)

Nordinonediol (**15**, 3 mg) was treated with acetic anhydride (3 mL) and pyridine (1.5 mL). The reaction mixture was stirred overnight at room temperature then poured over water-ice (20 mL). The suspension was extracted with ethyl acetate (3×10 mL), and the organic extract worked up in the usual manner to yield 2.7 mg of crude product. Purification of the crude by dry flash column chromatography (ethyl ether-Skellysolve B 3:1, packed in a dispo pipette) afforded O,O,O,O-tetraacetylnordinonediol (**K'**, 1.2 mg, yield = 27%). TLC: R_f 0.23

(ethyl ether-hexane 3:1, 2×). IR ($CHCl_3$ cast): 2920, 1774, 1736, 1370, 1259, 1022 cm^{-1}. HRMS: m/z calcd. for $C_{26}H_{32}O_{11}$ (M^+): 520.1944; found: 520.1947(18), 478(100), 460(6), 436(9), 418(25), 376(4), 358(21), 316(7), 150(82).

Nordinonediol (**15**) from monocillin V (**6**)

Monocillin V (**6**, 4.5 mg) was dissolved in a mixture of water-acetone (1:1, 3 mL), and the mixture acidified with perchloric acid (70%, 1 drop). The reaction was left at room temperature overnight then poured over water (15 mL) and extracted with dichloromethane (3×10 mL). The organic layer was separated, dried (anh. sodium sulfate), and concentrated to yield 3.5 mg (74.4%) of a yellowish crude identified as nordinonediol (**15**) on the basis of its spectroscopic data.

Preparation of monorden (**1**) by chlorination of monocillin I (**2**)

Monocillin I (**2**, 25 mg) was dissolved in dimethylformamide (DMF, 0.5 mL). N-Chlorosuccinamide (NCS, 10.1 mg) in DMF (0.5 mL) was added, and the mixture allowed to stir at room temperature for 48 h. The reaction mixture was poured into water (20 mL). The resulting suspension was extracted with dichloromethane (3×15 mL) and the organic layer washed with water (4×15 mL). The extract was dried (anh. sodium sulfate) and concentrated under reduced pressure to yield the crude product (23.7 mg). The TLC of the product showed the presence of two major compounds. These were separated by preparative TLC (20×20 cm, ethyl ether-Skellysolve B 3:1, 3×) and identified as monocillin I (**2**, less polar) and monorden (**1**, more polar) by comparison of their TLC behavior and spectroscopic ddata with those of authentic samples.

Bromination of monocillin I (**2**)

Monocillin I (**2**, 10.5 mg) was dissolved in DMF (0.5 mL) and to this a solution of N-bromosuccinamide (NBS, 4.3 mg) in DMF (0.5 mL) was added. The reaction mixture was stirred at room temperature and the course of the reaction was followed by TLC. When no major change could be observed (24 h) the mixture was poured over water (20 mL), and extracted with dichloromethane (4×10 mL). The organic layer was washed with water (2×40 mL), dried (anh. sodium sulfate), and concentrated to give crude product (24.3 mg). The resulting mixture showed three components by TLC. The least polar component corresponds to monocillin I (**2**), while the other two components were presumed to be brominated derivatives. Monocillin I (**2**, 3 mg) was purified by dry flash column chromatography (ethyl ether-Skellysolve B 60:40, 4 in long, 0.4 cm diameter). The brominated derivatives were purified by preparative TLC (10×10 cm, ethyl ether-Skellysolve B 55:45, 7×). The less polar of the two was identified as the dibromo derivative of monocillin I (**17**, 1.5 mg). TLC: R_f0.36 (ethyl ether-Skellysolve B 60:40, 3×). ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ7.36 (1H, dd, 10, 16 Hz), 6.22 (1H, dt, 2, 9 Hz), 5.86 (1H, dd, 2.5, 11 Hz), 5.52 (1H, m), 4.70 (1H, d, 17 Hz), 3.98 (1H, d, 17 Hz), 3.18 (1H, bs), 2.94 (1H, dt, 2, 9 Hz), 2.38 (1H, dt, 3, 15 Hz), 1.54 (3H, d, 7 Hz). HRMS: m/z calcd. for $C_{18}H_{16}O_6{}^{79}Br^{81}Br$ (M^+): 487.9293; found: 487.9280(13), 308(87).

The other component corresponded to the monobrominated derivative of monocillin I (**116**, 2 mg). TLC: R_f 0.25 (ethyl ether-Skellysolve B 60:40, 3×). ^1H NMR (200 MHz, $C\underline{D}Cl_3$): δ7.42 (1H, dd, 10, 16 Hz), 6.66 (1H, bs), 6.18 (1H, t, 8 Hz), 5.85 (1H, bd, 8 Hz), 5.50 (1H, m), 4.78 (1H, bd, 17 Hz), 3.94 (1H, bd, 17 Hz), 3.17 (1H, bs), 2.94 (1H, bd, 9 Hz), 2.37 (1H, bd, 16 Hz), 1.24 (3H, d, 8 Hz). HRMS: m/z calcd. for $C_{18}H_{17}O_6{}^{81}Br$ and $C_{18}H_{17}O_6{}^{79}Br$ (M^+): 410.0188 and 408.0208; found: 410.0217(23), 408.0211(22), 230(87), 228(89).

Methylation of monocillin I (**2**)

Monocillin I (5 mg) was mixed with acetone (2 mL), potassium carbonate (10 mg) and methyl iodide (0.1 mL). The resulting mixture was heated under reflux for 2 h, then cooled and filtered. The solvent was evaporated to give a yellow oil (7.1 mg) which shows two components on

TLC. Purification of the mixture was carried out by preparative TLC (10×10 cm, ethyl ether-Skellysolve B 60:40, 5×). The least polar component was identified as monocillin I-monomethyl ether (**23**, 2.6 mg). TLC: R_f0.53 (ethyl ether-Skellysolve B 60:40, 3×). IR ($CHCl_3$ cast): 3400, 2920, 2840, 1650, 1615, 1258, 1160 cm^{-1}. ^1H NMR (400 MHz, $C\underline{D}Cl_3$): δ11.40 (1H, s), 7.82 (1H, dd, 12, 18 Hz), 6.40 (2H, m), 6.26 (1H, t, 12 Hz), 6.04 (1H, d, 16 Hz), 5.93 (1H, dd, 3, 10 Hz), 5.59 (1H, m), 5.20 (1H, d, 14 Hz), 3.79 (3H, s), 3.63 (1H, d, 14 Hz), 3.27 (1H, bs), 3.11 (1H, dt, 2, 10 Hz), 2.44 (1H, dt, 3, 16 Hz), 1.64 (3H, d, 7 Hz). HRMS: m/z calcd. for$C_{19}H_{20}O_6$ (M^+): 344.1260; found: 344.1280(58), 164(100).

The more polar compound was identified as monocillin I-dimethyl ether (**22**, 0.9 mg). TLC: R_f 0.46 (ethyl ether-Skellysolve B 60:40, 3×). IR ($CHCl_3$ cast): 2953, 1720, 1610, 1420, 1460, 1261 cm^{-1}. HRMS: m/z calcd. for $C_{20}H_{22}O_6$ (M^+): 358.1416; found: 358.1427(49), 178(100).

O,O-Diacetylmonocillin I (**26**)

A mixture of monocillin I (**2**, 4.9 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) was allowed to stir overnight at room temperature. The reaction mixture was poured over water (20 mL) and extracted with ethyl acetate (3×10 mL). The organic extract was dried (anh. sodium sulfate) and concentrated. The crude product (13 mg) was purified by passing it through a small bed of silica gel (flash) packed in a dispo pipette. Pure O,O-diacetylmonocillin I (**26**, 3.7 mg, yield = 60%) was obtained as a milky oil. TLC: R_f0.38 (ethyl ether-Skellysolve B 60:40, 3×). IR ($CHCl_3$ cast): 1773, 1730, 1620, 1610, 1430, 1370 cm^{-1}. HRMS: m/z calcd. for $C_{22}H_{22}O_8$ (M^+): 414.1315; found: 414.1328(12), 372(9), 330(5), 234(11), 192(51), 150(100).

Bromination of monocillin I-monomethyl ether (**23**)

The monomethyl derivative of monocillin I (**23**, 0.9 mg) was dissolved in DMF (0.25 mL) and a solution of NBS (0.5 mg) in DMF (0.25 mL) was added. The reaction was allowed to stand over a weekend (60 h). The mixture was poured over water (10 mL) and extracted with dichloromethane (4×10 mL). The organic layer was separated, washed with water (2×20 mL), dried (anh. sodium sulfate), and concentrated to give a yellowish crude (8.6 mg) which showed two spots on TLC. The mixture was purified by preparative TLC (10×10 cm, ethyl ether-Skellysolve B 55:45). The less polar component was identified as the starting material (**23**, 0.3 mg). The other component proved to be a mixture of the corresponding mono- and dibromo-derivatives. TLC: R_f 0.42 (ethyl ether-Skellysolve 60:40). HRMS: 502(13), 424(36), 322(31), 245(8).

Purification of the brominated mixture was repeated. Preparative TLC (10×10 cm, chloroform, 3×) afforded each component in pure form. The less polar component was identified as the monobrominated derivative. TLC: R_f 0.67 (chloroform, 2×). HRMS: m/z calcd. for $C_{19}H_{19}O_6{}^{81}Br$ and $C_{19}H_{19}O_6{}^{79}Br$ (M^+): 424.0344 and 422.0365; found: 424.0348(26), 422.0366(25), 244(68), 242(67).

The dibromo-derivative was found to be the more polar compound. TLC: R_f 0.59 (chloroform, 2×). HRMS: m/z calcd. for $C_{19}H_{18}O_6{}^{79}Br^{81}Br$ (M^+): 501.9450; found: 501.9477(27), 322(100).

Appendix of Experimental

Potato Dextrose Broth (PDB)

24 g Difco potato dextrose broth.

0.048 g yeast extract.

Suspend in 1 L of water and warm slightly to dissolve completely.

Potato Dextrose Agar (PDA)

39 g Difco potato dextrose agar.

Dissolve in 1 L of water.

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